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REGULATION OF IMMEDIATE EARLY AND EPITHELIAL-MESENCHYMAL TRANSITION GENES EXPRESSION BY ADIPOCYTOKINES IN THE FEMALE REPRODUCTIVE TRACT

Zhufeng Yang
University of Nebraska-Lincoln, yzhufeng@yahoo.com

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by

Zhufeng Yang

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Zhufeng Yang M.S.

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Advisor: Jennifer R. Wood

Individuals who are overweight or obese are at increased risk for cancer. However, the mechanistic link between obesity and cancer is poorly defined. Adipose tissue produces hormones and pro-inflammatory cytokines with mitogenic properties. Many of these hormones and cytokines are altered in obese individuals and may lead to disruption of the normal balance between cell proliferation, differentiation, and apoptosis. Thus, the objective of this study was to determine how adipocyte-derived factors regulate the expression of genes that contribute to cell proliferation and migration. To compare immediate early gene (cell proliferation) and epithelial-mesenchymal transition (cell migration) gene expression between untreated and treated cells, HeLa cells were exposed to IGF-1 (100ng/ml), leptin (100ng/ml), TNFα (10ng/ml), or IL-6 (10ng/ml) and QPCR analyses were carried out. Immediate early gene expression was regulated by all four hormones. Specifically, IGF-1 increased Jun, Fos, and Il-8; leptin increased Jun and Il-6; IL-6 increased Fos, Il-6, and Il-8; and TNFα increased Jun, Fos, Il-6, and Il-8 mRNA abundance. Genes that regulate the epithelial-mesenchymal transition were also

regulated by IGF-1, IL-6, and TNF. Specifically, IGF-1 increased Snail1 and Snail2; IL-6 increased Snail1; and TNF increased Jag1 mRNAs. Interestingly, the expression of Snail1, Snail2, JUN, and FOS was increased in the uterus of age-matched obese compared to normal-weight mice. Western blot analyses demonstrated that these changes in mRNA abundance were associated with increased phosphorylation of Akt, Erk1/2, Jnk, and Stat3 in treated compared to untreated cells suggesting that these signaling factors play a role in the regulation of immediate early and epithelial-mesenchymal transition gene expression. These studies demonstrate for the first time a mechanistic link between factors produced and secreted by adipocytes and the expression of genes associated with cell transformation, proliferation, and migration. These cell functions play an important role in tumorigenesis and therefore changes in their expression may provide a plausible mechanism for obesity-dependent increases in a myriad of cancers.

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CHAPTER 1

LITERATURE REVIEW

Clinical correlations between BMI and Cancer Risks

Obesity is a worldwide epidemic and health threat which arose as an unintended consequence of economic, social, and technological advances. Obesity is defined based on the body mass index (BMI) measurement which is calculated by the following formula: BMI = weight (kg)/height squared (m^2). BMI provides an accurate measurement to determine whether a patient is obese or overweight (1, 2) . A person is defined as overweight if BMI is greater than 25.0 but less than 29.9 and as obese if BMI is greater than 30.0. Since 1980, over one billion adults have been classified as overweight or obese (3, 4). Likewise, the CDC indicated that in 2007-2008 (5), approximately 72.5 million adults in the United States were obese (CDC, unpublished data, 2010). Unfortunately, obesity is growing at an exponential rate in both developed and developing countries, and is even expanding among youth. In the United States, the ratio of people classified as obese is more than one-third of all adults.

Patients who are obese experience several health problems which impact the quality of life. Specifically, obese adults are at increased risk for development of coronary heart disease, hypertension, stroke, and type 2 diabetes. In addition, overweight and obesity is associated with certain types of cancer including kidney, breast (postmenopausal), endometrium, colon, gallbladder, ovary, pancreas and esophagus (6), (7). For example, after menopause, the risk for breast cancer is 1.5 times greater in an obese compared to an age-matched, normal-weight woman (8, 9). Likewise, Bergstrom *et al.* reported that obese women have up to a four-fold increased risk for endometrial

cancer than normal-weight women (10). In fact, approximately 40 percent of endometrial cancer patients are overweight or obese. Increased cancer risk due to obesity also impacts men. For example, there is a weak positive relationship between male BMI and the risk for colon cancer (11).

Despite the plethora of data linking obesity to increased cancer incidence, the mechanism of obesity-induced cancer development and progression is still unclear. Furthermore, it is likely that the specific mechanisms differ depending on cancer type. Adipose tissue is an endocrine organ and therefore, one possible mechanism includes chronic exposure to altered hormone and adipocytokine profiles associated with obesity (12, 13). Excess adipose tissue is characterized by altered endocrine hormone synthesis and increased macrophage infiltration which is an important source of pro-inflammatory cytokines produced by this tissue. Specifically, adipose tissue produces interleukin 6 (IL-6), tumor necrosis factor alpha (TNFα), adiponectin, inhibitor of NF-κB kinase b (IKKb), macrophage migration inhibitory factor, nerve growth factor, vascular endothelial growth factor (VEGF), plasminogen activator inhibitor 1 and haptoglobin, which act at both the local (autocrine/paracrine) and systemic (endocrine) level (14-16). Furthermore, chronic, excess adipose tissue alters the synthesis of endocrine hormones including sex steroids, insulin, and insulin-like growth factor (IGF).

Hormone Profiles and Obesity

IGF System: Insulin-like growth factors (IGFs) are polypeptide hormones which are mainly produced by liver but are also synthesized by various other tissues including the ovary and uterus. The IGF family consists of two ligands, IGF-1 and IGF-2, which both share approximately 50% structural homology to insulin (17). The synthesis of IGF-

1 in the liver is stimulated by growth hormone (GH) and represents a primary mediator of GH action (18). Conversely, the synthesis of IGF-2 is GH-independent. Both IGF-1 and IGF-2 play an important role in the regulation of cell growth and survival via regulation of mitogenesis, differentiation, and apoptosis (19, 20). Thus, IGF-1 and IGF-2 have growth-promoting effects on almost every cell in the body, especially skeletal muscle, cartilage, bone, liver, kidney, nerves, skin, hematopoietic cell, and lungs, although the actions of IGF-2 are likely restricted to fetal development in mammals (19, 20).

The actions of the IGF ligands are mediated by one of three receptors, the IGF-1 receptor, the IGF-2 receptor and the insulin receptor. The IGF-1 receptor (IGF1R) is a tyrosine kinase receptor which includes two α and two β subunits that have 60%, sequence homology to the insulin receptor (Fig. 1.1) (19). Both IGF-1 and IGF-2 bind with high affinity to the IGF1R which is expressed in all tissues except the liver. Activation of the IGF1R promotes cell survival, cell proliferation, protein synthesis, and DNA and RNA synthesis (19, 20). The IGF-2 receptor (IGF2R) is monomeric and structurally identical to the mannose 6 phosphate receptor (Fig. 1.1). IGF-2 binds to the IGF2R with much higher affinity than IGF-1. Furthermore, the IGF2R does not have tyrosine kinase activity and acts as a decoy receptor which promotes degradation and therefore clearance of IGF2 from the circulation (19). In addition to these two IGF receptors, IGF-1 and IGF-2 are able to bind to the insulin receptor, although at a much lower affinity (i.e. 100-fold less than insulin). However, studies have demonstrated that high concentrations of IGF-1 can induce insulin receptor dependent signaling (17, 21). The ability of IGFs to bind to these receptors is also dependent of the expression of binding proteins. There are 6 binding proteins (IGFBP1 – IGFBP6) which interact with

both IGF-1 and IGF-2 and inhibit interaction of ligands with receptors as well as prolong ligand half-life (19). Interestingly, IGFBP-1, IGFBP-3, and IGFBP-5 can also potentiate IGF activity.

The effect of an obese phenotype on the GH-IGF axis remains unclear with conflicting results documented in the literature. The concentration of serum total IGF shows little or nodiurnal variation by Baxter (22). However, Frystyk et al. showed that obesity is associated with a decrease in GH. The level of GH secretion is diminished in obesity and reversible with weight loss. However, obesity-dependent decreased GH level is not always correlated with decreased IGF-1 level in obesity (23, 24). In obesity, free IGF-1 levels are actually increased in obese men and to a lesser extent in woman (Fig. 1.2) (25). In addition, individuals with low IGF-1 level tend to more easily weight gain and obesity (23, 24, 26). Using a mouse model of obesity (Lethal Yellow; LY), our lab has demonstrated changes in total circulating IGF-1 levels during the development of obesity (Fig. 1.3) (Mack and Wood, unpublished data). Specifically, at 6 weeks of age, prior to the onset of obesity, circulating levels of IGF-1 were significantly increased ($P < 0.01$) in LY female mice compared to age-matched female C57BL/6 (B6) control. These increased levels decreased in LY mice returning to comparable levels to B6 mice by 12 weeks of age (Mack and Wood, unpublished data). Studies have also shown an association between circulating IGF-1 levels and bady fat in domestic livestock. For example, the development of lower perirenal fat observed in low body weight involves an alteration of the IGF system as shown in the liver and skeletal muscle of growth-retarded fetuses and neonates in pig (27, 28). Furthermore, these changes in IGF-1 concentrations have an effect on reproductive performance in beef cattle (29).

The relationship between IGF1 and neoplasia has drawn attention for several years. In 1987, Tricoli reported that increased IGF-1 mRNA is increased 3 to 5 fold in human colon cancer compared to the control (30). When IGF-1 was added to serum free media with the concentration of 10µg/L, the growth of mouse colon adenocarcinoma increased (31). Moreover, a high IGF-1/IGFBP-3 ratio would increase the risk of colon cancer development suggesting that IGF-1 stimulates the growth of colon cancer cells (32). In addition, IGF-1 has mitogenic effect on the St16, St42 and MKN45 gastric cancer cell lines , as well as human pancreatic cell line such as ASPC-1and COLO357 (33, 34). In lung cancer cells, there is increased IGF-1 concentration in the cancerous tissue, and IGF-1 stimulates the growth of the lung cancer cell line CALU-6 inducing a 52% increase (35). In breast cancer cells, IGF-I and IGF-II are potent mitogens, and combine with estrogen to stimulate cell growth in a synergistic manner (36). Ferlanetto reported that IGF-1 stimulated DNA synthesis in multiple breast cancer cell lines including MCF-7, T47D, MDA-MB-23 and HBL-100 (36). Clinical studies also demonstrated that there is elevated IGF-1 levels in breast cancer patients (37). Finally, men with elevated IGF-1 concentrations are at increased risk for developing prostate cancer (37).

Taken together, these data provide evidence that the IGF system is involved in cancer growth. One mechanism of IGF-dependent tumorigenesis may be that IGFs are mitogenic and anti-apoptotic peptides that enhance the proliferation and reduce the death of various cell types. Angiogenesis also plays a critical role in the development of cancers. IGF-I is able to activate sphingosine kinase (SphK) in human endothelial cells, leading to sphingosine-1-phosphate (S1P) formation. Insulin-like growth factor binding

protein-3 induces angiogenesis through IGF-I- and SphK1-dependent mechanisms. There is also evidence that the increase of breast tumor angiogenesis depends on interactions of M6P/IGF-IIR with mannose 6-phosphate-containing proteins (38). IGFs also play important roles in cancer metastasis. Recent studies showed that numerous predictive markers of colorectal carcinoma metastasis include IGF-II, TGF-a, EGFR, matrix metalloproteinase (MMP)-2, vascular endothelial growth factor (VEGF) (17). In melanoma patients, there is a significant correlation between high IGF-IR level and the risk of liver metastasis. Furthermore, overexpression of IGF-IR in the primary gastric tumor is correlated with increased lymph node metastasis (39-41). Finally, high levels of IGF-1 and IGF-2 enhance choriocarcinoma cell adhesion and invasion (39-41). Taken together, these collective studies provides strong evidence that the IGF system is involved in human cancer progression and emphasizes the importance of targeting IGF signaling and developing drugs capable of inhibiting IGF signaling as anticancer therapy (42, 43).

Leptin: Leptin is a peptide hormone of 16kDa and 167 amino acids. It is one of the most important adipose derived hormones, which plays a key role in regulating energy intake and energy expenditure, and regulating appetite and metabolism. Leptin is the product of the "obesity" (ob) gene which was discovered in 1994 by Zhang and is located on chromosome 7 in humans (44). Leptin is expressed primarily in the adipocytes of white adipose tissue and, at lower levels, in gastric epithelium and placenta (45, 46). Interestingly, like other endocrine hormones, leptin levels fluctuate in a diurnal and pulsatile fashion. Circulating leptin levels reach maximum after midnight, whereas they

are lowest around noon. There is also evidence that glucocorticoids up-regulate leptin levels in the human (47, 48)

Leptin interacts with six types of receptors including Ob-Ra–Ob-Rf, or LepRa-LepRf. Leptin receptors have two isoforms, long and short, with the short form generally located in peripheral tissue including the liver, kidney, heart, skeletal muscles, and the pancreas, and the long form mainly expressed in the hypothalamus. The main function of leptin in the hypothalamic neurons is to regulate appetite control. Specifically, leptin reduces food intake by upregulating anorexigenic (appetite-reducing) neuropeptides, such as α-melanocyte-stimulating hormone, and downregulating orexigenic factors, primarily neuropeptide Y (NPY) in the hypothalamus (47, 48).

In obese individuals circulating leptin levels are generally higher than in normal-weight individuals. These higher leptin concentrations in the serum of obese individuals is associated with both increased fat mass and increased leptin release from larger adipocytes (49). Unlike IGF-1, there is a significant correlation between leptin levels and percent body fat in both sexes. However, the absolute peak leptin level is higher in women than in men (49, 50). In ruminant animals, leptin level in serum fluctuates with body condition in a similar pattern as humans. Furthermore, leptin levels are associated with different nutritional conditions in cattle. For example, plasma leptin concentration measured 4 h after meal is positively related to feed intake and to plasma 3-OH-butyrate, and negatively related to plasma NEFA. Leptin concentrations decrease after meal intake in well-fed cows but increase in underfed cows (51, 52). In our mouse model of obesity, leptin is significantly increased ($P < 0.001$) in LY compared to B6 females at 12 and 24 weeks of age (Fig 1.3; Mack and Wood, unpublished data). Based on these data, leptin

was originally considered a good target for treating obesity by the scientific and clinical communities. However, the complexity of the leptin axis and the development of leptin resistance make leptin a poor target for obesity therapy.

Given that obesity is risk factor in various cancers and leptin plays a significant role in obesity, the relationship between leptin levels and cancer risk has been seriously considered. Several reports demonstrate a role for leptin in the pathogenesis of a series of different forms of cancers, including cancers of the male and female reproductive tract (breast, endometrial, ovarian and prostate cancer), cancers of the gastrointestinal tract (esophagus, gastric and colon cancer) and leukemias (49). In breast cancer, leptin increases cell proliferation and cell transformation (anchorage-independent growth). One mechanism for these cellular changes is leptin-dependent increases in AP-1 activation which results in increased cdk2, cyclinD1 and P450 aromatase expression and hyperphosphorylation of the retinoblastoma (Rb) protein. Furthermore, it induces c-myc expression and stabilizes ERα expression (53, 54). In colorectal cancer, leptin increases cell growth through the ERK1/2 pathway, increases cell invasion via an PI-3K, Rho- and Rac-dependent pathways, and reduces cell apoptosis via stimulation of NF-kB signaling (55). In prostate cancer, leptin increases cell proliferation and suppresses apoptosis (56). In pancreatic cancer, leptin decreases cell proliferation through stimulation of STAT3 and STAT5b phosphorylation (57). In ovarian and lung cancer, leptin increases proliferation through the ERK1/2 pathway (58, 59).

IL-6 and TNFα: Adipose tissue is not only the source of endocrine hormones, but is also infiltrated with macrophages which are an important source of inflammatory factors. In fact, excess adipose tissue associated with obesity is recognized as a chronic

inflammatory organ characterized by high expression of interleukin 6 (IL-6) , tumor necrosis factor alpha (TNFα), inhibitor of NF-κB kinase b (IKKb), macrophage migration inhibitory factor, nerve growth factor, vascular endothelial growth factor, plasminogen activator inhibitor 1 and haptoglobin; increased acute-phase reactants, and activation of inflammatory signaling pathways (15, 16). For example, adipose tissue is a significant source of circulating TNFα in most rodent models of obesity as well as human cases of obesity (60, 61). IL-6 is also produced by human adipose tissue and its circulating levels increase with the increased value of BMI (62, 63).

Interleukin 6 and TNFα are multifunctional cytokines that are traditionally involved in the regulation of the immune response, hematopoiesis, and inflammation. Interleukin-6 is secreted by T cells and macrophages in response to trauma and mainly acts as a B cell differentiation factor. Interleukin-6 binds to the sIL-6 receptor (gp80, present either on the cell surface or in solution) resulting in dimerization of gp80 and the transmembrane gp130 and the activation of Janus kinase (JAK)(64). Tumor necrosis factor a belongs to a superfamily of ligands which regulate the establishment of multicellular structures such as lymphoid organs, hair follicles, bone, and lactating mammary gland (65). Upon stimulation, macrophages produce TNFα which is a 26-kDa transmembrane protein that is cleaved into a 17-kDa biologically active protein. Biologically active TNFα binds to one of two receptors, 55-kd (TNFR I) or 75-kd (TNFR II), which are found on most cells. The interaction of biologically active TNFα with these receptors results in multiple cellular responses including apoptosis (most cells), cell proliferation (lymphocytes), bone resorption, and insulin resistance (Fig. 1.4) (66).

Cytokines are known to have both tumor-promoting and inhibitory effects with these opposing effects dependent on their relative concentrations and the presence of other factors (67). Furthermore, inflammation is recognized to play important roles in the pathogenesis of many types of malignancies. Given the ability of adipose cells to produce cytokines especially during obesity, there could be an association between the pro-inflammatory cytokine produced by adipose cells and the increased risk of cancer in obesity. Indeed, elevated circulating IL-6 levels have been reported in breast cancer patients indicating that IL-6 levels may represent an early marker of breast tumors (67, 68). In addition, Grivennikov and Bollrath have demonstrated the contribution of interleukin-6 and its downstream effector STAT3 on colitis-associated colon cancer (69, 70). Conversely, TNFα generally inhibits tumorigenesis and viral replication. Recently, TNFα has been used as a regional treatment of locally advanced soft tissue sarcomas and metastatic melanomas and other tumors (71). Together with chemotherapeutic drugs, TNFα shows a synergistic antitumor effect (72). However, TNF-α alone induced only a mild central necrosis with no objective tumor response observed. In rat models, chemotherapeutic drugs with TNF-α improved the accumulation of selectively tumor up to 3 to 6 fold (71, 72). In addition, to these roles of IL-6 and TNFα, other bioactive peptides produced the adipose cell promote angiogenesis associated with metastasis including vascular endothelial growth factor, hepatocyte growth factor, and heparin-binding epidermal growth factor-like growth factor (68).

Signaling Pathways Regulated by Adiopocytokines

The interaction of adipocytokine ligands with their receptors stimulates multiple signal transduction pathways. Figure 1.5 shows the complex signaling network

(including cross-talk between pathways) that functions downstream of the insulin receptor (IR), the IGF-1 receptor, TNFα receptor, IL-6 receptor and leptin receptor (73).

IGF-1 dependent signaling: When the IGF-1R heterotetramer is activated, the IRS-1 complex is recruited and phosphorylated, resulting in several signal transduction cascades to be activated including the phosphotidylinositol-3 kinase (PI3K)/Akt pathway (Fig. 1.5). Specifically, the lipid products of the PI3-kinase activates a cascade of PI (3, 4, 5)-phosphate (PIP3)-dependent serine/threonine kinases including PDK1. Substrates of this kinase include PKC isoforms, the serum and glucocorticoid-inducible kinase SGK and the product of the Akt proto-oncogene. The activation of Akt is an important and central mediator of IGF-1action on glucose metabolism, cell proliferation and differentiation, and protein synthesis. In addition to Akt, IGF-1 activation of PI3K can stimulate c-jun N-terminal kinase (JNK/SAPK) resulting in increased activity of the AP-1 transcription factors (74). For example, Poulaki et al demonstrated that systemic inhibition of IGF-I signaling with a receptor-neutralizing antibody, or with inhibitors of PI-3 kinase (PI-3K), c-Jun kinase (JNK), or Akt suppressed retinal Akt, JNK and AP-1 activity. In addition, intravenous administration of IGF-I increased retinal Akt, JNK and AP-1 activity (74). One mechanism that low IGF-I decreases cancer is that reduced circulating IGF-I level attenuates activation of Akt and mTOR signaling pathways (73, 75).

Leptin- and IL-6 dependent signaling: Leptin interaction with its receptors including Ob-Ra–Ob-Rf, or LepRa-LepRf activates the Jak-Stat and mitogen activated protein kinase (MAPK) signal transduction pathways (73, 76)(77). Mostly, Leptin binding to the extracellular domain of the long form of the leptin receptor (LRb)

which is crucial for leptin action and mainly expressed at basomedial hypothalamus, then activates the JAK2 tyrosine kinase which associates with LRb via the Box 1 motif and intracellular amino acids 31–36 of LRb (78). The long form of the receptor is crucial for leptin action and mainly expressed at basomedial hypothalamus (79). Short form may relate to leptin across the blood-brain barrier. The three primary intracellular signaling pathways emanate from LRb, including JAK2-Tyr 1138-stat3 pathway, Tyr 985 - SHP2 - ERK pathway and JAK2 tyrosine phosphorylation sites (78). Activation of JAK2 results in interaction with a conserved SH2 domain containing protein and phosphorylation and dimerization of STATs (Fig.1.5) (73). Suppressors of cytokine signaling (SOCS) are recently identified inhibitors of the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway (79). Increase in SOCS mRNA is observed when cells are exposed to a wide range of cytokines or growth factors , such as IL-4, IL-6, GH, Leptin (80). In fact, the phosphorylated STAT dimer translocates to the nucleus to activate target genes and SOCS transcription. SOCS-1 and SOCS-3 inhibit cytokine signaling in a feedback manner by inhibiting JAK or by inhibiting the activated receptor complex (79).

IL-6 interaction with its receptor also activates the JAK/STAT signaling pathway. Specifically, IL-6 binds to α-receptor subunits of IL-6R (gp 80) which are not involved in the intracellular signal-transduction cascade. After ligand binding, the cytokine and gp 80 complex are able to efficiently dimerize with gp 130 which is the signal-transducing receptor component (81, 82), activate JAK and recruit STAT family members (Figure 1.6)(64). Activation of JAK also results in the recruitment of SH2 domain protein, and

subsequently increased activity of the mitogen-activated protein kinase (MAPK) cascade. Thus, MAPK cascade is another important pathway stimulated by IL-6 (73, 83).

TNFα dependent signaling: TNFα exerts its biological action through activation of TNFRI and II which in turn activate the extracellular signal-regulated kinase (ERK-1/2), p38 mitogen-activated protein kinase (p38 MAPK), phosphoinositide 3-kinase (PI3K)/Akt and nuclear factor-κB (NF-κB) pathways. TNFRI initiates the majority of TNF's function. The binding of TNF to TNF-R1 results in the activation of two major transcription factors, nuclear factor κB (NF-κB) and c-Jun. which is responsible for the inducible expression of genes important for diverse biological processes, including cell growth and death, development, oncogenesis, and immune, inflammatory, and stress responses (66). The chemical inhibitor LY294002 which blocks PI3K activity inhibits TNFα-induced Akt and ERK phosphorylation. Likewise, the IKK-2 inhibitor IMD-0354 readily prevents TNFα-induced IκB-α phosphorylation and degradation, and thus activates NFkB activation. In addition, TNFα is able to induce IL-6 expression via TNFRI, which is dependent on p38 MAPK. Activation of IL-6 mRNA expression by TNF-α, can also be regulated by JNK via PI3K/Akt/NF-kappaB axis (73, 84). Tumor necrosis factor receptor-associated factors (TRAFS) are adaptor proteins that couple the tumor necrosis factor receptor family to signaling pathways. TRAF2, TRAF5 and TRAF6 have been demonstrated to mediate activation of NF-kB and JNK, which is important pathway of TNFa (85).

Transcription Factors activated by Adipocytokine Signaling

AP-1 transcription factors: According to Liu's report, IGF-1 also activated the AP-1 (86). AP-1 converts extracellular signals into cell and make changes of expression

of specific target genes which harbour AP-1 binding site(s) in their promoter or enhancer regions. The family of AP-1 transcription factors includes Fos, Jun and ATF families of proteins (86-88). Normally, these AP-1 factors bind to the12-O-tetradecanoylphorbol-13-acetate (TPA) response elements (5′-TGAG/CTCA-3′) or the cAMP response elements (CRE,5′-TGACGTCA-3′)(86). AP-1 activity is regulated by interactions with other transcriptional regulators and mediated by upstream kinases which link AP-1 to various signal pathways including MAPK and JNK (86). AP-1 transcription factors are involved in a large variety of biological processes such as cell differentiation, proliferation, apoptosis and oncogenic transformation. For example, Jochum et al. show that c-Jun binds to the promoter of the P53 and cyclin D1, and then inhibit the p53 gene expression and stimulate the cycline D1 gene expression during mouse development and in a tumorigenesis model (Fig. 1.7). C-Fos and FosB also binds to the promoter of the cyclinD1 and stimulate the gene expression of cyclinD1. JunD can bind to the promoter of Ras and inhibit the gene expression of Ras, while Jun B binds to the promoter of p16 and stimulates the gene expression of p16 (86). AP-1 transcription factors play important role in cell differentiation, proliferation, apoptosis and oncogenic transformation.

FOXO transcription factors: In addition to AP-1, several other transcription factor families are the target of signaling pathways activated by adipocytokines including FOXO family, NFκB family, and Stat family. FOXO (Forkhead box o) transcription factors play crucial roles in cell growth, proliferation, differentition, metabolism and stress tolerance. FOXO protein sequence contains 80 to 100 amino acids, which form a forkhead motif that looks like butterfly in loops and is also called the winged helix motif that binds to DNA. They belong to helix-turn-helix class of proteins. Four members of

FOXO family have been discovered. They are FOXO1, FOXO3, FOXO4 and FOXO6 (89, 90). FOXO activity is modified post-translationally due to phosphorylation, acetylation and ubiquitination. For example, Akt/PKB signaling inhibits FOXO activity through phosphorylation which causes them to be translocated out of the nucleus (91). FOXO1 is able to interact with TSC2, androgen receptor, estrogen receptor alpha, and CREB binding protein (92, 93). FOXO3 functions as a trigger for apoptosis through activation of Bim and PUMA, or down-regulation of anti-apoptotic proteins FLIP (94). FOXO3 is known as a tumour suppressor, and inhibition of FOXO3 tends to enhance tumorigenesis (95).

NF-κB transcription factors: NF-κB is a protein complex of transcription factors, with the name nuclear factor kappa-light-chain-enhancer of activated B cells. NF-κB family consists of 5 members: RelA, RelB, c-Rel NF-κB1 and NF-κB2, which all contain a Rel domain in their N-terminus. The NF-κB1 and NF-κB2 proteins are derived from precursor proteins p105 and p100 respectively, through selective degradation of C-terminal ankyrin repeat region by the ubiquitin/proteasome pathway. NF-κB is widely distributed in all animal cell types. Its functions are involved in cellular responses to stress, cytokines, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens (96). The kappa light chains of NF-κB are critical components of immunoglobulins allows NF-κB to be crucial in the regulation of the immune responses. Generally, inactivated NF-κB is located in the cytosol bound by the inhibitor IκBα. Once activated by certain stimuli (e.g. leptin) (97), IκB kinase (IKK) is activated and is able to phosphorylate the IκBα protein, leading to its ubiquitination, which results in the separation of IκBα from NF-κB, and further degradation of IκBα by the proteosome. As a

consequence, the activated NF-κB is translocated into the nucleus. Active NF-κB is able to bind to the promoters of target genes, and initiate gene transcription (98). IGF-1 is able to keep sustained activation of NF-κB, but independent of IKK phosphorylation (99). In addition, NF-κB can be activated by TNF-α via phosphatidylcholine-specific phospholipase C-induced "acidic" sphingomyelin breakdown (100). TNFα is also able to activate NF-kB through MAPK and caspase pathways leading to apoptosis. Interestingly, both TNFα and IL-1 activate NFκB using MAPK signaling pathway in a synergistic manner (101).

STAT transcription factors: Signal transducer and activator of transcription proteins (STATs) are transcription factors, which play crucial roles in cell growth, survival and differentiation. There are seven members in STAT family: STAT1, STAT2, STAT3, STAT5a, STAT5b, and STAT6, which are activated by JAK. Janus kinase phosphorylates specific tyrosine residues in the STAT protein. When the phosphorylated dimers of STAT are activated, they are transported in the nucleus through importing a/b and RanGDP complex. In the nucleus, STAT dimers activate transcription of target genes by binding to their promoter regions of genes at gamma activated site (GAS) motif. The STAT proteins are inactivated once they are de-phosphorylated by nuclear phosphatases (102, 103). Dysregulation of the STAT pathway is involved in the formation of primary tumours and leads to increased angiogenesis, enhanced survival of tumours and immunosuppression (102, 103).

Epithelial Mesenchymal Transition

Epithelial Mesenchymal Transition (EMT) is a normal developmental process characterized by inhibition of E-cadherin expression, loss of cell adhesion and increase of cell mobility. There are three types of EMTs (104, 105). Type 1 EMT is seen in primitive epithelial cells transitioning to motile mesenchymal cells during gastrulation and in primitive neuroepithelial cells which form migrating neural crest cells. Type 1 EMT is also associated with implantation. Type 2 EMT takes place during secondary epithelial or endothelial cells transition to resident tissue fibroblasts and is involved in wound healing, tissue regeneration, and organ fibrosis. Thus, both Type 1 and Type 2 EMT are associated with normal biological functions during development and wound repair, respectively. Conversely, type 3 EMT occurs in neoplastic cells and is involved in carcinoma cell invasion and metastasis. Carcinoma cells undergoing a type 3 EMT invade and metastasize and thereby the primary nodules transition to metastatic tumor cells and migrate by blood to form secondary distant nodules (106). Clarification of EMT process at the molecular level would help to understand mechanisms of cancer progression, detection of cancer metastases, and therapeutic intervention. There is a variety of biomarkers to use to detect all three subtypes of EMT (Table 1.1) (105), including cell-surface markers, cytoskeletal markers, extracellular matrix proteins, transcription factors and micro RNAs.

Snail: The Snail family of zinc finger transcription factors, Snail1 and Snail2, are inducers of the EMT. Snail genes are expressed in all EMT processes resulting in the conversion of cells from epithelial to mesenchymal with migratory properties. Thus, Snail-induced EMT is involved in formation of tissues and organs during embryonic

development and also the invasion and metastasis of carcinoma cells during tumorigenesis. In addition, Snail genes also act as survival factors and inducers of cell movement independent of the induction of EMT. Snail-induced EMT is mostly due to the repression of E-cadherin gene expression. Snail also regulates other aspects of the EMT such as suppressing cyclin D protein , cyclin-dependent kinase 4 and the expression of caspase, DNA fragmentation factor, Bcl-interaction death agonist, increasing MMP expression., and increasing expression of mesenchymal cell marker such as fibronectin and vitrionectin (105). Interestingly, pro-inflammatory cytokines and hormones released by adipose tissue are associated with Snail stabilization. In Snail expression knockdown inflammation-mediated breast cancer metastasis model, it shows the inhibition of inflammatory cytokines induced EMT. Snail can be stabilized by the inflammatory cytokine TNFα through activation of the NF-kappaB pathway (107).

E-cadherin: Cadherin-1 (CDH1, E-cadherin), also known as CD324, is a tumor suppressor gene (108). In epithelial tissues, E-cadherin is localized on the surfaces of epithelial cells at adherent junctions and plays an important role in cell to cell adhesion (109). Specifically, calcium-dependent interactions among E-cadherin molecules are important to mediate and maintain the adherent junctions of epithelial cell-cell contact (110). In cancer cells, the function of E-cadherin is associated with the dedifferentiation/ aggressiveness of tumours and the transition from transformed cells to the invasive phenotype. In fact, E-cadherin acts as an invasion-suppressor gene. Loss of E-cadherin is considered to be diagnostic of a poor clinical prognosis with benign lesions to invasive, metastatic cancer. In addition, CDH1 knockout promotes cancer cell proliferation,

invasion and metastasis, including gastric, breast, colorectal, thyroid, and ovarian cancers (111).

ZEB1: Zinc finger E-box binding homeobox 1 (ZEB1), binds to E-box-like elements that overlap with those bound by Snail family transcription factors. ZEB1 is normally expressed in proliferating mesenchymal and neural progenitors. In fact, ZEB is EMT-inducer and transcriptional repressor which promotes invasion and metastasis (112). ZEB1 is a crucial promoter of metastasis and inhibits expression of the microRNA-200 (miR-200) family. ZEB1 links EMT-activation by suppressing stemness-inhibiting microRNAs (miRNAs) and thereby acts as a promoter of mobile, migrating cancer stem cells (112)(111). In cancer, overexpression of ZEB1 is associated with repression of E-cadherin. ZEB1 is expressed in most of poorly differentiated pancreatic cancers and in invasive, undifferentiated tumor cells of pancreatic adenocarcinomas. Heterozygous mutation of ZEB1 also leads to craniofacial abnormalities such as cleft secondary palate and defective nasal formation. In a knockdown mouse model, decreased ZEB1 leads to an epithelial transition without increased expression of E-cadherin and reduces not only cancer cell invasion and metastasis, but also the number of tumor-bearing mice and the average size of primary tumors (111).

Markers of EMT

Acquired markers		Attenuated markers	
Name	**EMT type**	**Name**	**EMT type**
Cell-surface proteins			
N-cadherin	1, 2	E-cadherin	1, 2, 3
OB-cadherin	3	ZO-1	1, 2, 3
α5β1 integrin	1, 3		
αVβ6 integrin	1, 3		
Syndecan-1	1, 3		
Cytoskeletal markers			
FSP1	1, 2, 3	Cytokeratin	1, 2, 3
α-SMA	2, 3		
Vimentin	1, 2		
β-Catenin	1, 2, 3		
ECM proteins			
α1(I) collagen	1, 3	α1(IV) collagen	1, 2, 3
α1(III) collagen	1, 3	Laminin 1	1, 2, 3
Fibronectin	1, 2		
Laminin 5	1, 2		
Transcription factors			
Snail1 (Snail)	1, 2, 3		
Snail2 (Slug)	1, 2, 3		
ZEB1	1, 2, 3		
CBF-A/KAP-1 complex	2, 3		
Twist	1, 2, 3		
LEF-1	1, 2, 3		
Ets-1	1, 2, 3		
FOXC2	1, 2		
Goosecoid	1, 2		
MicroRNAs			
miR10b	2	Mir-200 family	2
miR-21	2, 3		

Table 1.1. Biomarkers for epithelial-mesenchymal transitions. Adapted from (105).

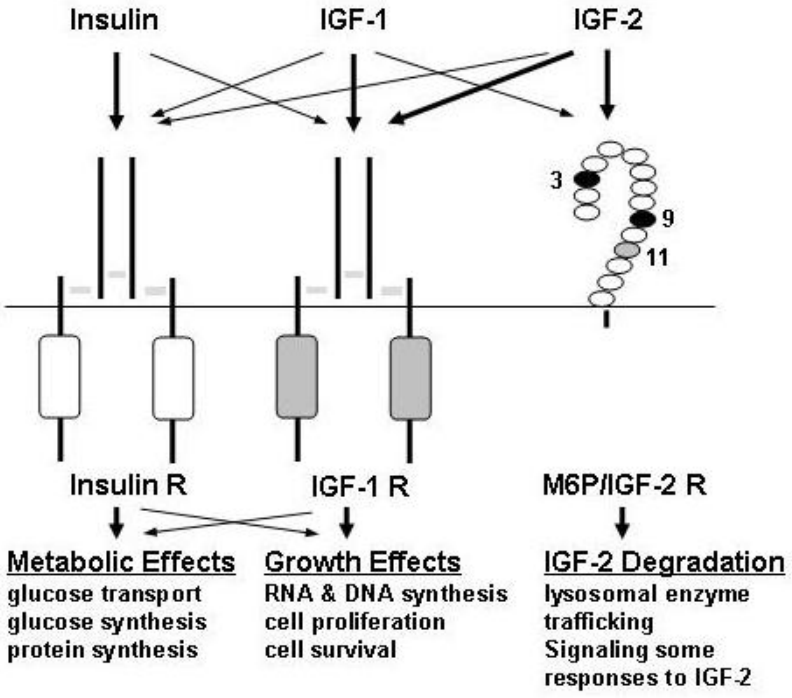

Figure 1.1. IGF-1 and IGF-2 interact with the IGF-1 receptor (IGF-1 R), the IGF-2 receptor (IGF-2 R) and insulin receptor (insulin R). IGF1-R and insulin R are tyrosine kinase receptors that mediate overlapping functions of the IGF ligands. The IGF-2 R is structurally identical to the mannose 6 phosphate (M6P) receptor and promotes degradation of the ligand. Adapted from (19).

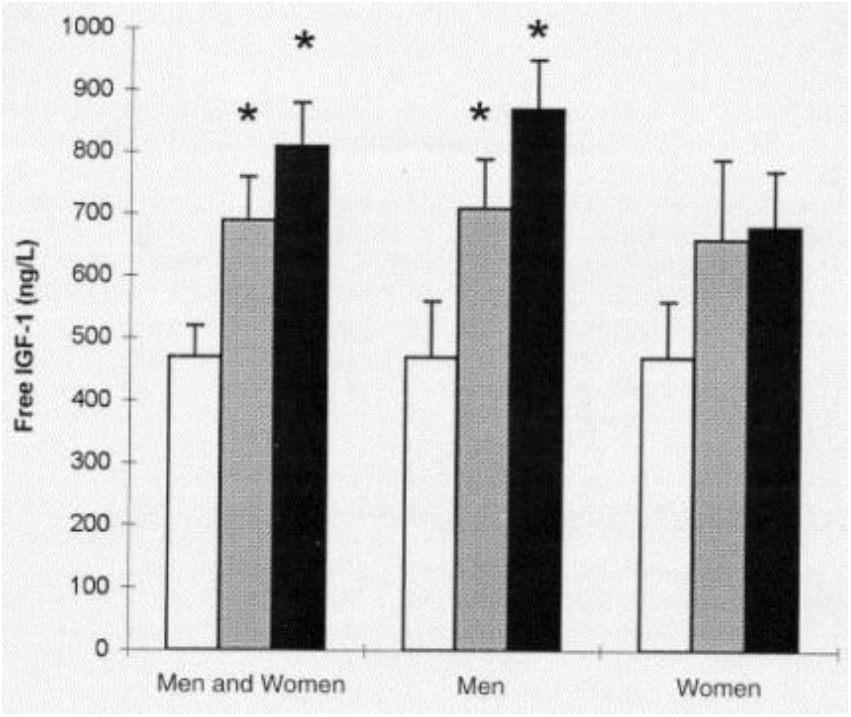

Figure 1.2. Free IGF-1 levels in lean and obese men and women. There is an increased free IGF-1 level in obesity, However, less elevated free IGF-1 level in woman compare to man. The white bars are lean, grey bars are overweight, and black bars are obese The white bar is controls (BMI < 25), while the gray bar show moderate obesity (25 < BMI< 30), and black bar shows severe obesity(BMI>30). Adapted from (25).

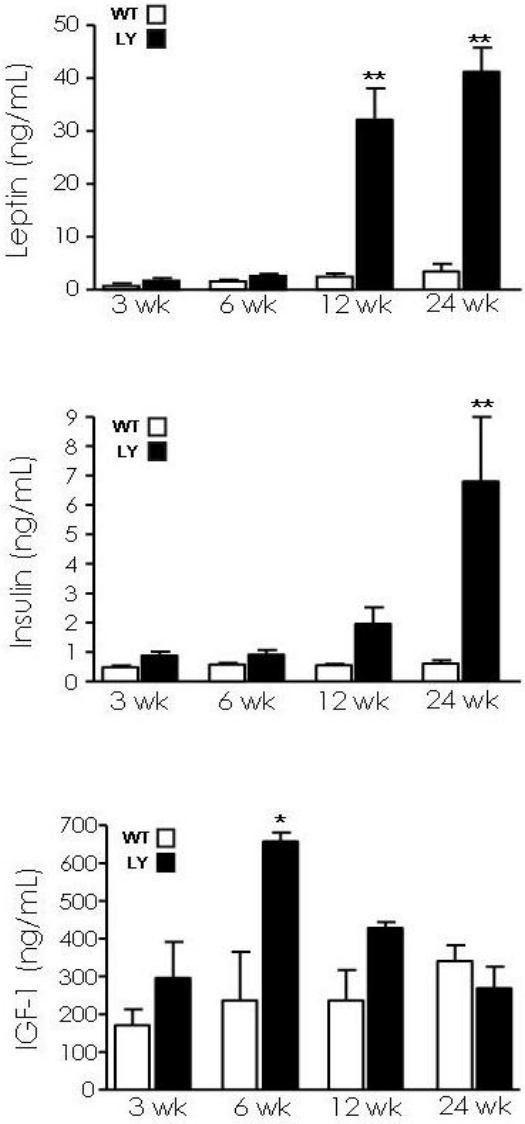

Figure 1.3. Circulating levels of leptin (ng/ml), insulin (ng/ml), and IGF-1 (ng/ml) in 3, 6, 12, and 24 week old female B6 and LY mice. Circulating levels of leptin (ng/ml), insulin (ng/ml), and IGF-1 (ng/ml) were measured by ELISA using blood serum collected from 3, 6, 12, and 24 week old B6 (white bar) and LY mice (black bar). (Mack and Wood, unpublished data)

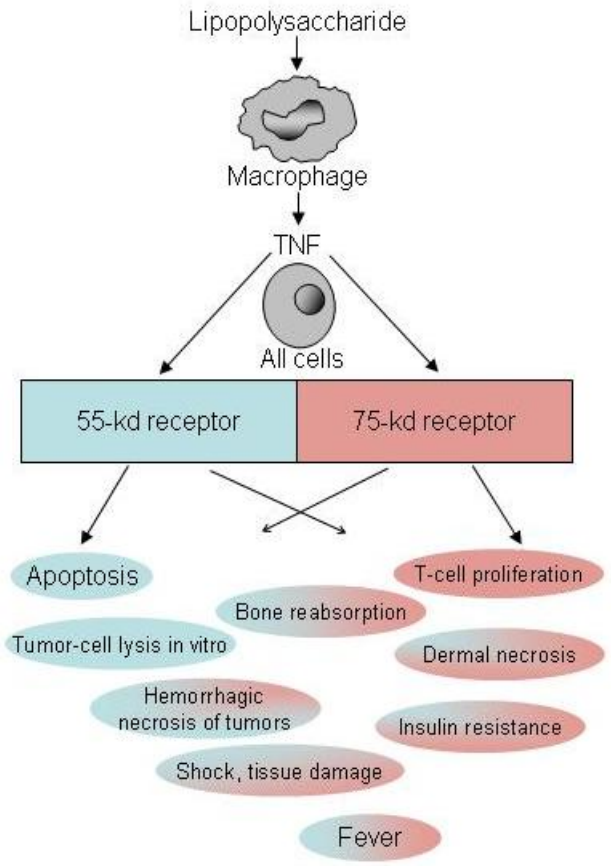

Figure 1.4. TNF signal transduction pathway. TNFa is responsible for the inducible expression of genes important for diverse biological processes, including cell growth and death, development, oncogenesis, and immune, inflammatory, and stress responses. Adapted from (66).

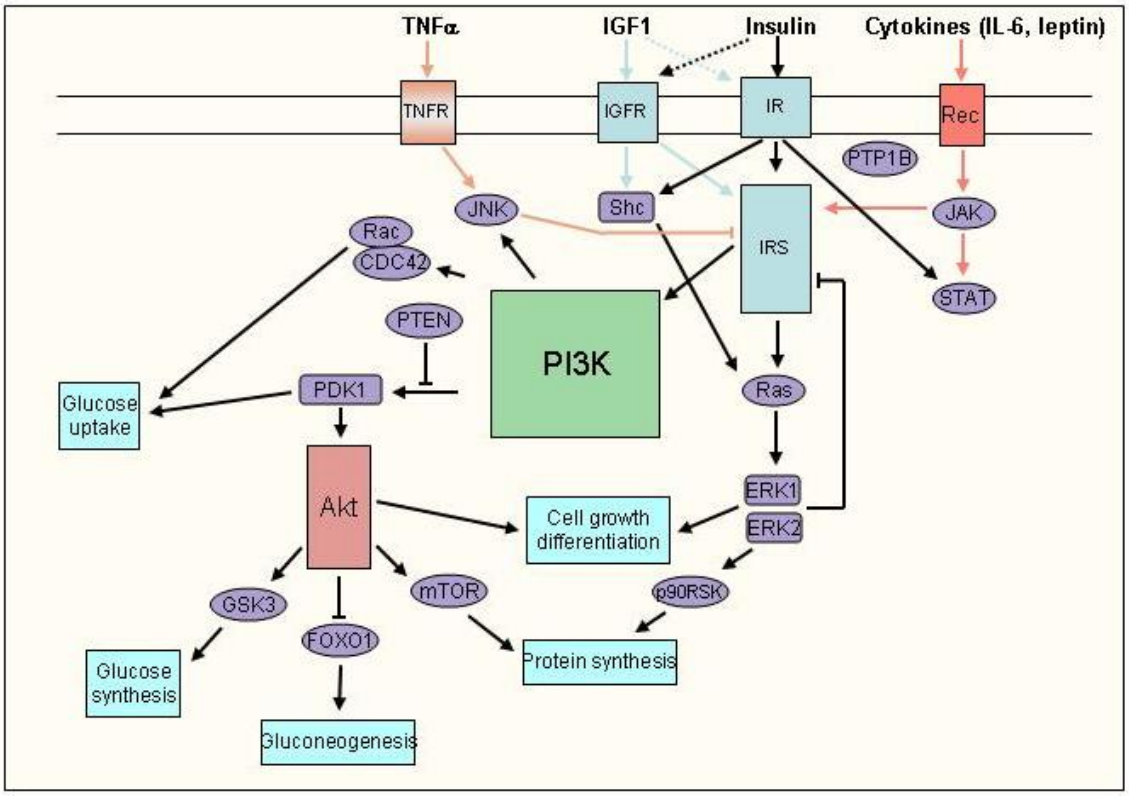

Figure 1.5. IGF-1, leptin, Il-6 and TNFa signal transduction pathways. Upon binding to their receptors, all activate the protein kinase signaling. The phosphorylation would cause several signal transduction cascades to be activated including (ERK1 and ERK2), forkhead box O1 (FOXO1), glycogen synthase kinase 3 (GSK3), Janus kinase (JAK), c-Jun-N-terminal kinase (JNK). Adapted from (73).

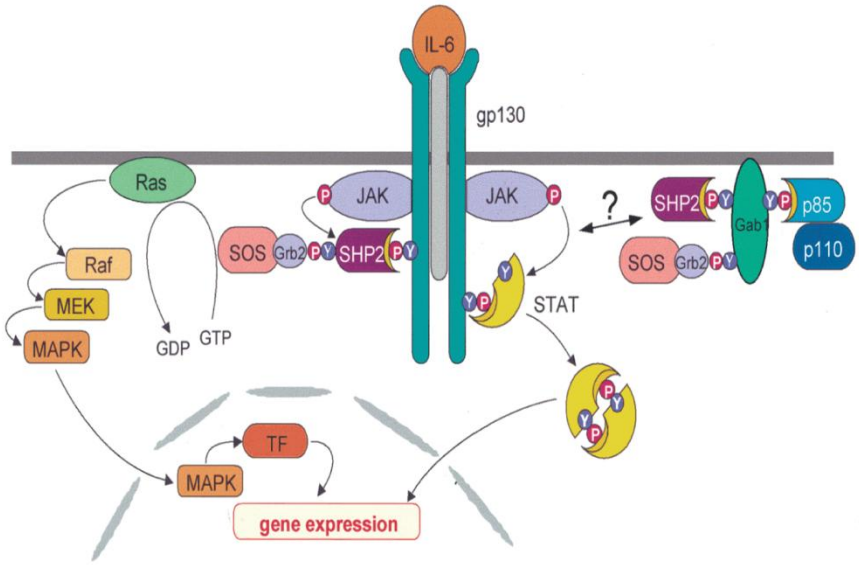

Figure 1.6. IL-6 activates the JAK/STAT pathway and the MAPK cascade. IL-6 binds to plasma membrane receptor complexes containing the common signal transducing receptor chain gp 130 (glycoprotein 130). Signal transduction involves the activation of JAK (Janus kinase) tyrosine kinase family members, leading to the activation of transcription factors of the STAT (signal transducers and activators of transcription) family. Another major signalling pathway for IL-6-type cytokines is the MAPK (mitogen-activated protein kinase) cascade. Taken from (64).

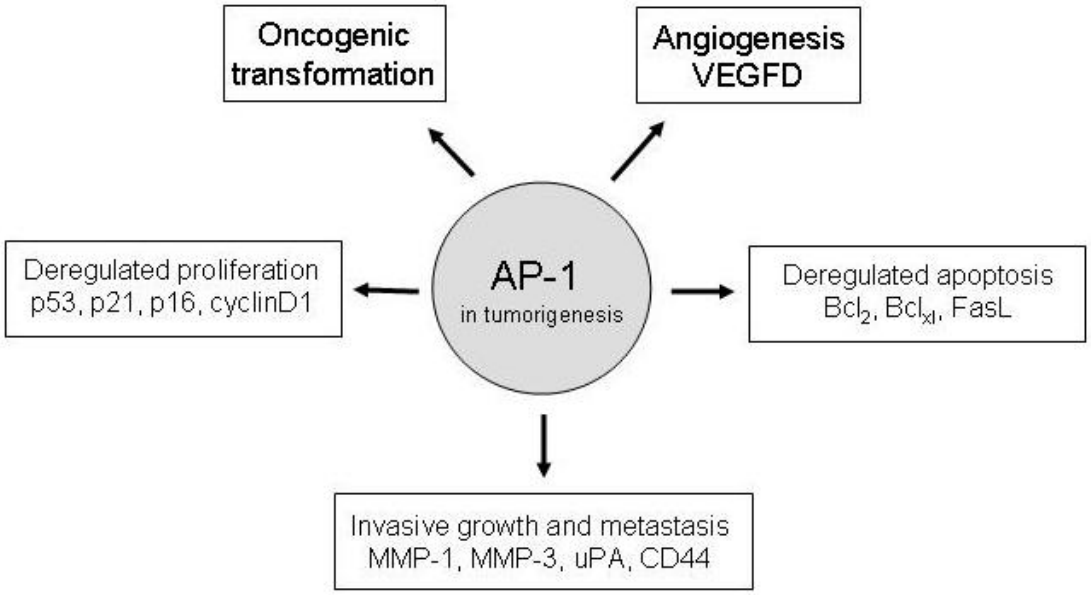

Figure1. 7. AP-1 functions in multistep tumorigenesis. AP-1 proteins regulate oncogenic transformation, proliferation, apoptosis, invasive growth and angiogenesis through modulating the expression of genes. Adapted from (86).

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CHAPTER 2

Regulation of Immediate Early and EMT Markers Genes by IGF-1 in HeLa Cells and the Lethal Yellow Mouse Uterus

Abstract

IGF-1 can regulate differentiation, apoptosis, cell growth and survival. IGF-1 levels fluctuate during the development and progression of obesity. Furthermore, IGF-1 signaling has been implicated in abnormal cell growth associated with tumorigenesis. Thus, these studies suggest a potential link between obesity-induced abnormalities in IGF-1 levels and the incidence of cancer. The objective of this study was to identify IGF-1 dependent changes in the expression of genes that regulate cell proliferation and migration. In this study, HeLa cells were treated with 100ng/ml IGF-1 for 0-24 hour and RT-QPCR was carried out using primers against immediate early (IE) genes which regulate cell proliferation and differentiation and markers of the epithelial-mesenchymal transition (EMT) which promote cell invasion and migration. IGF-1 stimulated a significant transient increase in JUN, FOS, IL-8, IL-6 and CASP10. Furthermore, SNAI1, SNAI2 and JAG1 mRNA abundance was significantly increased by IGF-1. Interestingly, we showed that *JUN, SNAI1* and *SNAI2* mRNA abundance is increased in the uterus of our obese mouse model when circulating IGF-1 levels are increased. The signaling pathways of AKT, ERK1/2, STAT3, and JNK likely play a role in IGF-1 dependent regulation of IE and EMT gene expression. To assess the activity of IGF-1, cells were treated for 0-60 minutes. Western blot analyses demonstrated the increased phosphorylation of AKT. Furthermore, IGF-1 dependent phosphorylation of ERK1/2, STAT3, and SPAK/JNK occurred at 15min post treatment. Thus, this study has identified

a novel mechanism for IGF-1 dependent regulation of cell movement and survival, which has important implications for the process of tumorigenesis.

Introduction

Obesity has been recognized as a national health threat and a major public health challenge. Since 1980, over one billion adults considered to be overweight or obese (1) (2). In 2007--2008, according to measured weights and heights (3), approximately 72.5 million adults in the United States were obese (CDC, unpublished data, 2010). Obese adults are at increased risk for many diseases, such as coronary heart disease, hypertension, stroke, type 2 diabetes, and certain types of cancer. But the mechanism by which obesity increases the risk of cancer is unclear. In obesity, the enlarged amounts of adipose tissue results in the distortion of the normal balance of several endogenous hormones.

The insulin-like growth factor (IGF-1) 1 is a metabolic hormone that fluctuates with nutrient availability. IGF-1 was once thought to be produced only by the liver and dependent on growth hormone (GH) (4). Stimulation of the liver (and other tissues) by GH causes the production of IGF-1. Obesity is associated with a decrease in GH. Free fatty acids released in response to GH secretion may feedback on the hypothalamus or pituitary as a mechanism to limit GH release (5, 6). Numerous investigators have observed GH secretion to be diminished in obesity and reversible with weight loss (5, 6). In vivo, protein deficiency also decreases IGF-1 levels. Likewise, nutritional repletion causes increased IGF-1 production. It is reported that IGF-1 resistance becomes evident in obese individuals similar to insulin and leptin resistance. Furthermore, in obesity free

IGF-1 levels are actually increased in obese men and to a lesser extent in woman, suggesting GH-independed changes in IGF-1 synthesis (7). IGF-1 is now known to be produced in a wide variety of organs and tissues, especially produced by adipose tissue (4). The increased amounts of IGF-I could be secreted from the excessively enlarged amounts of adipose tissue (8). In addition, free fatty acids may increased sensitivity to feedback effects of IGF-1 or increased free IGF-1 concentrations in obesity (7, 9). The increased free IGF-1 may affect cell growth and development which is related to tumorigenesis. The current study suggests a relationship between increased IGF-1 and cancer tumorigenesis.

In the current study, the objective is to identify IGF-1 dependent changes in the expression of genes associated with cell transformation and/or migration. To address this objective, two models were used, an immortalized cell line and a mouse model of obesity. HeLa cells are an immortal cell line used in scientific research, including cancer research, AIDS, the effects of radiation and toxic substances (10). The HeLa cell which was derived from cervical carcinoma, is a good in vitro model for immortal, grows indefinitely. The LY (LY, A^y/a) mouse has a gene deletion between the promoter and first exon region of the agouti gene, which results in ectopic over expression of the agouti protein (11, 12). The over expressed agouti binds to melanocortin-4 receptors (MCR4) as an antagonist of α-MSH in hypothalamus and in the skin. In hypothalamus, the over expressed agouti interferes with normal satiety control and causes over-eating (figure 2.6.). In the skin, it results in a yellow coat color. Thus, the LY (LY, A^y/a) mouse is model can be used to mimic the mechanism of obesity development in humans. Furthermore, previous data from our lab shows the increased IGF-1 levels in the 6 week

old LY mouse compared to the wild-type C57BL/6 control mouse (Mack and Wood, unpublished data). With the increased IGF-1 level and overweight, the LY is good vivo model which can be used to determine the relationship between obesity dependent increasing IGF-1 and the expression of genes associated with cancer tumorigenesis.

MATERIALS AND METHODS

HeLa cell culture and whole cell protein extracts: 500,000 HeLa cells (American Type Culture Collection, Manassas, VA) were cultured in 10 cm dish with Eagle's Minimum Essential Medium pH 7.4 (Sigma, St. Louis, MO), containing 1.5 g/L NaHCO3, 0.11 g/L Sodium pyruvate, 0.292 g/L L-glutamine, 10 ml/L Penn-strep, 1 ml/L phenol red and 100 ml/L of heat inactivated FBS (Hyclone, Logan, UT, USA). After 24h, the cells were changed to the same medium except there was no FBS included for serum starvation. After 24h serum starvation, cells were treated with 100ng/ml IGF-1 (Cell Signaling Technology, Danvers, MA) for 0, 15, 30 or 60 minutes. The cells were washed with 1X HBSS (Invitrogen, Carlsbad, CA, USA) and cells collected in RIPA buffer (150 mM NaCl, 1 mM EDTA, 50mM Tris-HCl pH7.4, 1% NP-40, 0.25% Na-deoxycholate) containing phosphatase inhibitors (1 mM NaF and 1 mM Na3VO4) and Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics). Cells in the RIPA buffer were sonicated 15 seconds and then centrifuged at 10,000 x g for 5 minute to separate cell debris and the supernatant which contain soluble protein. The protein concentration of each experimental replicate was determined with the Pierce BCA Protein Assay (Rockford, IL, USA).

HeLa cell culture and RNA extraction: 200,000 HeLa cells (American Type Culture Collection, Manassas, VA) were cultured in 3.5 cm dish and serum starved for 24 h as described above. After serum starvation, cells were treated with 100ng/ml IGF-1 (Sigma, St. Louis, MO) for 0, 0.25, 0.5, 1, 2, 4, 8, 16 or 24 h. The cells were washed with 1X HBSS (Invitrogen, Carlsbad, CA, USA) and then collected in TRI reagent (Ambion Inc., Austin, TX, USA). Total RNA was extracted according to the manufacturer's protocol (Sigma, St. Louis, MO). After isolation, the total RNA was dissolved in 20 µl diethyl pyrocarbonate (DEPC) water. The RNA concentration was determined using Beckman Coulter DU 730 Life Science UV/Vis Spectrophotometer.

Reverse Transcription: Total HeLa cell RNA (5 µg) was mixed with 5 units of RQ1 RNase free DNase (Promega, Madison, WI), M-MLV RT buffer and DEPC water and incubated at 37°C for 30 minutes to remove genomic DNA contaminants. Before RT-PCR, 1.1 µl of RNA and RQ1 mix was removed from each sample to a new tube with 15 µl DEPC water, which should be used as No RT to detect the residual genomic DNA. The mixed RNA samples was subsequently combined with 2 µl Random primers (Promega), 2 µl dexoxynucleotide triphosphates (Fermantas; 10 mM dNTP mix), 2.4 µl DEPC water, and then were incubated in 65°C for 5 min. After that, the mixed samples were chilled on the ice quickly. 400 units of Moloney Murine Leukemia Virus reverse transcriptase (Promega), 3.2 µl of RT buffer (Invitrogen, Carlsbad, CA), and 2 µl 0.1 M DL-dithiothreitol (DTT) (Invitrogen, Carlsbad, CA) were added into each sample. The mixed samples were incubated at 37°C for 2 hour and followed by 15 min at 72°C to stop the reverse transcriptase. The cDNA was stored at -20°C for subsequent real time-PCR.

Quantitative, Real-Time PCR (qPCR) Analysis: Gene-specific forward and reverse primers were designed (Primer Express, Applied Biosystems, Foster City, CA) and synthesized (Integrated DNA Technologies, Coralville, IA) and store at -20°C. The information of the primers and probes are shown in table 2.1 and table 2.2. Each set of gene-specific primers was tested to determine the maximal concentration of primers that could be used to produce specific amplification of the target sequence without primer dimer amplification. Before use, each cDNA sample was diluted 1:10. Glyceradehyde-3-phosphate dehydrogenase (Gapdh) gene is a housekeeping gene and used as a control for reverse transcription efficiency and well-to-well variation in template. PCR was performed with 10 µl Taqman Universal Master Mix (Applied Biosystem, Foster City, CA), 1 µl cDNA, 1 µl probe with primers, and 8 µl DEPC to make up the reaction volume to 20 µl. QRT-PCR was performed in 364 well plates (Axygen Scientific, Union City, CA) with an adhesive cover film (VWR, Scientific Products, North Kansas City, MO) in 7900HT Fast Real-Time PCR system (Applied Biosystems). Quantitative PCR (qPCR) reactions of the other target genes were carried out using 1:10 dilutions of each cDNA sample and standard samples with Power SYBR Green PCR Master Mix (Applied Biosystem, Foster City, CA). All experimental and Gapdh PCRs were carried out in separate wells in triplicate. These relative values are plotted against the threshold value for each dilution to generate a standard curve. An arbitrary value of template was assigned to the highest standard and corresponding values were assigned to the subsequent dilutions. According to the slope and y-intercept of the standard curve, 7900HT Fast Real-Time PCR system assigned a value of the relative amount for each

experimental and Gapdh triplicate. The value of the average of the experimental triplicate divided by the average of the Gapdh triplicate was used for statistical analysis.

Western Blot Analyses: The protein samples were resolved by SDS-polyacrylamide 10% gel electrophoresis which contains 4% stacking gel and 10% separating gel. Protein samples were loaded together with loading buffer and the separated protein was transferred to Immobilon PVDF (Millipore, Billerica, MA). Following transfer, the membranes were blocked with 5% nonfat dry milk in 1X TBST (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 01% (v/v) Tween 20) for 1 hour with gentle shaking in order to block the nonspecific proteins. After that, the blots were probed with the primary antibodies which are diluted in 5% w/v BSA, 1XTBS, 0.1% Tween 20. The membrane was then incubated with primary antibody against phospho-AKT (Cell Signaling Technology), phospho-ERK1/2 (Cell Signaling Technology), phospho-JNK, or phospho-STAT3 overnight at 4°C with gentle shaking. Blots were washed with 1X TBST and incubated for 1 hour with HRP-conjugated secondary antibody which was diluted with 5% nonfat dry milk in 1X TBST (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 01% (v/v) Tween 20). Proteins were incubated with West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) for 5 min and exposed to autoradiograph film (Fisher Scientific, Fairlawn, IL) in dark room. After the visualization of phosphorylated protein, the blots were stripped with Restore plus Western Blot Stripping Buffer (Pierce) in room temperature for 20 min and 37°C 10 min. Wash the blots with 1X TBST and blocked with 5% milk in 1XTBST. Next, incubated with primary antibody against total AKT, total ERK1/2, total JNK, or total STAT3 (Cell Signaling Technology) overnight at 4°C with gentle shaking. Total protein was exposed and visualized as described above. The

visualized total protein served as a loading control for each sample. The autoradiograph images films were scanned and the density of the protein band was determined in Photoshop. Semi-quantitative analysis of band density of the phosphorylated and total protein was calculated in each film as described by Miller (http://lukemiller.org/journal/2007/08/quantifying-western-blots-without.html). The amount of phosphorylated protein was normalized by total protein expressed in each sample. The normalized abundance of phosphorylated protein of each sample was subsequently compared to the untreated control sample. The data was showed as a fold-change.

Animals: C57BL/6 (B6; C57BL/6 a/a) and Lethal Yellow (LY; C57BL/6 Ay/a) female mice were used in the experiment, which the Founder mice were bought from Jackson Laboratory (Bar Harbor, ME, USA). All animal protocols were approved by the University of Nebraska Institute of Animal Care and Use Committee (IACUC) according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. According the animal protocols, all the Mice were fed with standard rodent chow and fresh water. The mice were housed in cages at virus-free room in a 12/12 hour light/dark cycle. Each cage owns no more than 5 mice.

Statistical Analyses: All statistical analyses were carried out by GraphPad Prism 4.0 (Graphpad Software, La Jolla, CA). QPCR data and Western blot data were analyzed using one-way ANOVA. Differences in data were considered statistically significant at $P < 0.05$. Different letters indicate significant differences in fold change in mRNA abundance compared to control for all qPCR data and phospho-protein/total protein ratio in time course. The Error bars represent mean ± SE.

Results

Significant gene expression differences In HeLa cells

To clarify the roles of IGF-1 in the regulation of immediate early genes, HeLa cells were treated with 100ng/ml IGF-1 in a time course after 24h serum starvation. *JUN* mRNA expression was elevated at 2 h compared to untreated control. The mRNA expression decreased gradually over time reaching control levels by 24 h. IGF-1 dependent increase in *FOS* was maxed at 0.25 hour and began to decrease quickly at 0.5 hour. For inflammatory response cytokines, IGF1 induced 5-fold increase of IL-8 mRNA at 4 h, but the decreased to control level at 16 h. IGF-1 caused fluctuations in MCP-1 and IL-6 over the time course evaluate but these changes were not significant. As an important pro-apoptotic factor, caspase 10 mRNA abundance was increased at 4 h to 1.5 times than control (Fig 2.2).

IGF-1 also regulates the mRNA abundance of markers of the epithelial-mesenchymal transition in HeLa cells (Fig. 2.3). IGF-1 induced mRNA expression level of *SNAI1* 2.5-fold at 2 h, compared to untreated control (Fig 2.3). SNAI2 mRNA was also increased by 3-fold but at an earlier time point (at 0.25 hour) and decreased back to control level by 1.5 h. IGF-1 did not change mRNA expression of ZEB2 and TWIST1. Furthermore, IGF-1 only had a modest effect on JAG1 mRNA abundance at 2-4 h post-treatment.

Significant gene expression differences between LY and age matched B6 controls.

To determine the potential role of altered IGF-1 levels in an obese mouse model on immediate early and epithelial-mesenchymal transition marker gene expression, RNA

was collected from uterus tissues of lethal yellow (LY) and C57BL/6 (B6) wild-type control mice. Fig 2.1 indicated that *Snai1*, *Snai2* and *Fos* have 1.5-fold increase in mRNA abundance in uterus tissues of LY compared to age-matched B6 mice. For Jun, mRNA abundance in LY uterus is 2 times higher compared to age-matched B6.

Signaling Pathways regulated by IGF-1 in HeLa cells

To further clarify the pathways that potentially mediate IGF-1 effects on the immediate early gene expression and epithelial-mesenchymal transition marker gene expression, we examined the phosphorylation of AKT and ERK1/2 in untreated and IGF-1 treated cells. In Fig 2.4., the level of AKT phosphorylation increased dramatically (5-fold) compared to the untreated control 15 min after the treatment of 100 ng/ml of IGF-1, and maintained this fold increase through the 1 hour time point. For ERK1/2, 100 ng/ml of IGF-1 induced phosphorylation by 3-fold by15 min, and interestingly, after that, the phosphorylation level of ERK dropped back to initial level by 30 minutes post-treatment. Fig 2.5 showed that 100 ng/ml of IGF-1 induced phosphorylation of pSAPK/JNK by 3-fold increase in 15 min, whereas after that, the phosphorylation level dropped back to initial level in another 15 minutes, while pSTAT3,its level increased by 2 fold in 15 minutes and kept dropping down in another 30 minutes.

Discussion

FOS and JUN belong to the AP-1 transcription factor family, which is involved in a large variety of biological processes such as cell differentiation, proliferation, apoptosis and oncogenic transformation. According to Liu's report, AP-1 activities were increased in the peritumoral tissue. AP-1 converts extracellular signals into cell and make changes

of expression of specific target genes which harbour AP-1 binding site(s) in their promoter or enhancer regions (13-15).

AP-1 proteins participate in tumorigenesis by regulating oncogenic transformation, proliferation, apoptosis, invasive growth and angiogenesis. Fos and FosB are dispensable for cell cycle progression, for example, fibroblasts and embryonic stem cells have no proliferation defect if lacking these components (16-18). In addition, Preston reported the overexpression of c-Fos induces cell death and resistant to apoptosis in an embryonic Syrian hamster cell line (19). However, c-Jun would act as both a positive and a negative modulator of apoptosis. In fact, dependent on its ability to heterodimerize with Jun proteins and to bind to DNA, c-Fos acts as oncogenic. Over-expressed c-Fos transforms chondroblasts and osteoblasts, and can therefore induce tumorigenesis (20, 21). Here, we demonstrated that IGF-1 is able to regulate the expression of FOS and JUN in HeLa cells. That the dramatic induction of JUN and FOS mRNA level caused by IGF-1, might open a new way to disclose the mechanism that IGF-1 uses activator protein 1 (AP-1) to turn on plenty of downstream target genes related to the initiation of tumorigenesis.

In mouse development and tumorigenesis model by Jochum's, C-Jun binds to the promoter of the P53 and Cyclin D1, and then inhibit the p53 gene expression and stimulate the cycline D1 gene expression. C-Fos and FosB also binds to the promoter of the CyclinD1 and stimulates the gene expression of CyclinD1 (15). Furthermore, in our mouse model, there is increased blood level of IGF-1 in the LY mouse compared to B6 mouse (Fig. 1.3). Circulating free IGF-1 levels are actually increased in obese men and to a lesser extent in woman (7). With the increased IGF-1 level and obesity, the LY (LY,

A^y/a) mouse is excellent vivo model for obesity. The increased IGF-1 could cause the unbalanced IGF-1 environment in the LY uterus. Moreover, the qPCR shows the increased mRNA expression of Fos in uterus in LY mouse at 6 weeks, which is the onset of obesity. This change is similar as IGF-1 treated in vitro model of HeLa cells. Therefore, our data suggest IGF-1's roles on cell growth, death, and tumorigenesis is possibly mediated by AP-1 pathway in obesity, which may suggest a new key to understand the relationship between obesity and reproductive tract cancer.

In our HeLa cells model, IGF-1 is able to induce an important inflammation factor IL-8 mRNA level with an increase of 4.5 fold in 4 h. Few data showed this fact. Therefore, this is a novel finding. IL-8 is a chemokine, secreted by macrophages, epithelial cells, endothelial cells, also by adipose cells. Increased expression of IL-8 and/or its receptors has been characterized in cancer cells by its contribution to cancer progression and metastasis (22, 23). IL-8 signaling promotes angiogenic responses in endothelial cells by increasing proliferation and survival of endothelial and cancer cells, and infiltrating neutrophils (22). The IGF-1 related increase of IL-8 increase the risk of cancer. Our data indicated that AP-1 subunits mRNA level could be increased by IGF-1 earlier than did IL-8. Consequently, AP-1 might possibly link a bridge between IGF-1 and IL-8.

The epithelial-mesenchymal transition (EMT) occurs not only during normal development but also in neoplastic cells which to promote invasion and metastasis (24). The snail family, SNAI1 and SNAI2, is important EMT makers. The zinc-finger transcription factor snail induced EMT involved in the invasion and metastasis of carcinoma cells during tumorigenesis. Perez-Mancera reported that Snail expression is

frequently upregulated in individuals with cancer (25). Our data demonstrated IGF-1 dependent increase in SNAI1 and SNAI2 mRNA in HeLa cells. Interestingly, there is also a significant increase of mRNA expression of SNAI1 and SNAI2 in LY mouse uterus in 6 weeks when compare to the B6 control mouse. Together with the increased IGF-1 level in the 6 week LY mouse, the increased SNAIL family in our vivo and in vitro model may show us another key to understand the obesity dependent unbalance IGF-1 level function in reproductive tract cancer.

Pro-inflammatory cytokines and hormones released by adipose tissue can generate a chronic inflammatory profile which may be associated with Snail protein stabilization. In Snail expression knockdown inflammation-mediated breast cancer metastasis model, it shows the inhibition of inflammatory cytokines reduced cell migration (26, 27). The increased IL-8 related to IGF-1 in HeLa cell may be associated to Snail family stabilization. Furthermore, there is a link between Snail expression and AP-1. In human skin keratinocytes, AP-1 is able to induce SNAIL expression in the exposure of Ultraviolet (UV) irradiation (28). Inhibition of AP-1 activity by over-expression of dominant-negative c-Jun extremely decreased Snail induction. Analyses of the Snail promoter revealed the presence of an AP-1 binding site. EMSA assay demonstrated that UV irradiation specifically induced c-Jun binding to this AP-1 site. Mutation of the AP-1-binding site completely blocked protein binding and inhibited UV irradiation-induced Snail promoter activity (27, 28). Thus, the increased AP-1 expression in obesity may cause not only increased tumorigenesis but also increased metastasis due to the AP-1 induced increase of Snail family protein. Together, these data suggest IGF-1, SNAI1/2

and FOS/JUN are important player in obesity and obesity related cancers. Clarification of their mechanism provides a promising way for the clinical therapy.

It is critical to figure out the mediating pathways which transfer IGF-1's signal. IGF-I stimulates phosphorylation of the IRS-1 complex , and then cause several signal transduction cascades to be activated including the PI3K pathway, which in turn activates the AKT pathway (29). Furthermore, low IGF-I decreases cancer due to attenuation of AKT and mTOR signaling pathways. Consequently, epidermal response to tumor promotion is diminished (29, 30). IGF-1 can stimulate AP-1 and c-jun N-terminal kinase (JNK) activation in retina according to Poulaki report (31). Using systemic inhibition of IGF-I signaling with a receptor-neutralizing antibody, or with inhibitors of PI-3 kinase (PI-3K), c-Jun kinase (JNK), or AKT, it shows a suppressed retinal AKT, JNK and AP-1 activity. In addition, intravitreous administration of IGF-I increased retinal AKT, JNK and AP-1 activity (31). Our western-blot data demonstrated that IGF-1 is sufficient to activate AKT, ERK, JNK, and STAT immediately at 15 minutes which is same as the report by Taniguchi. This provides a clue that AKT, ERK, JNK, and STAT are mediator to deliver IGF-1 signal to increase some downstream target gene expression, including regulation of IE and EMT gene expression. In the screening through chemical inhibitors, Snail induction is reduced by inhibitors of ERK and JNK.

IGF-1 is one of the important unbalanced hormones in obesity. While there are reports about decreased circulating IGF-1 levels or no difference in IGF-1 levels between obese and normal-weight individuals, our lab’s in vivo study show circulating levels of IGF-1 were significantly increased in the LY compared to B6 controls at 6 weeks. Together with the vitro data with IGF-1 treatment in HeLa cells, this study has identified

a novel mechanism for important relationship between obesity dependent IGF-1 and reproductive tract cancer. It is may be another key to understand a plausible mechanism for obesity-dependent increases in cancers of the female reproductive tract.

Table 2.1. Human Primer sequences used for QPCR analysis

Gene	Probe Dye	Primer	Sequence (5' to 3')
CASP10	SYBR Green	Forward	GGAGCTGTCTACTCTTCGGATGA
		Reverse	AGGGCTGTGAAGTGAGACATGAT
FOS	SYBR Green	Forward	CCTCGCCCGGCTTTG
		Reverse	GCCTCGTAGTCTGCGTTGAAG
JUN	SYBR Green	Forward	CTGGGAGGACCGGAGACA
		Reverse	GAGAAGCCTAAGACGCAGGAAA
MCP-1	SYBR Green	Forward	CAAGCAGAAGTGGGTTCAGGAT
		Reverse	TCTTCGGAGTTTGGGTTTGC
IL-6	SYBR Green	Forward	AGGGCTCTTCGGCAAATGTA
		Reverse	GAAGGAATGCCCATTAACAACAA
IL-8	SYBR Green	Forward	CTGGCCGTGGCTCTCTTG
		Reverse	CCTTGGCAAAACTGCACCTT
SNAI1	SYBR Green	Forward	CCCCAATCGGAAGCCTAACT
		Reverse	GCTGGAAGGTAAACTCTGGATTAGA
SNAI2	SYBR Green	Forward	CCTGGGCGCCCTGAA
		Reverse	TTCTCCCCCGTGTGAGTTCT
TWIST1	SYBR Green	Forward	TCCGCGTCCCACTAGCA
		Reverse	AGTTATCCAGCTCCAGAGTCTCTAGAC
ZEB2	SYBR Green	Forward	CCAGCTCGAGCGGCATA
		Reverse	GCCACACTCTGTGCATTTGAA
JAG1	SYBR Green	Forward	CAGCTCTGTGACAAAGATCTCAATTAC
		Reverse	AGGGCCTGTGTTGCTACAAGTT
GAPDH	Kit-Taqman Rodent GapDH Control Reagents Applied Biosystems (VIC Probe)		

Table 2.2. Mouse Primer sequences used for QPCR analysis

Gene	Probe Dye	Primer	Sequence (5' to 3')
Fos	SYBR Green	Forward	CCCCAAACTTCGACCATGAT
		Reverse	GGAGGATGACGCCTCGTAGTC
Jun	SYBR Green	Forward	CCGCCCCTGTCCCCTAT
		Reverse	TCCTCATGCGCTTCCTCTCT
Snai1	SYBR Green	Forward	GCCTGTGCCCGAACCTT
		Reverse	GCCAGACTCTTGGTGCTTGTG
Snai2	SYBR Green	Forward	GCCTGGGTGCCCTGAAG
		Reverse	TTGCAGACACAAGGCAATGTG
Gapdh	Kit-Taqman Rodent GapDH Control Reagents Applied Biosystems (VIC Probe)		

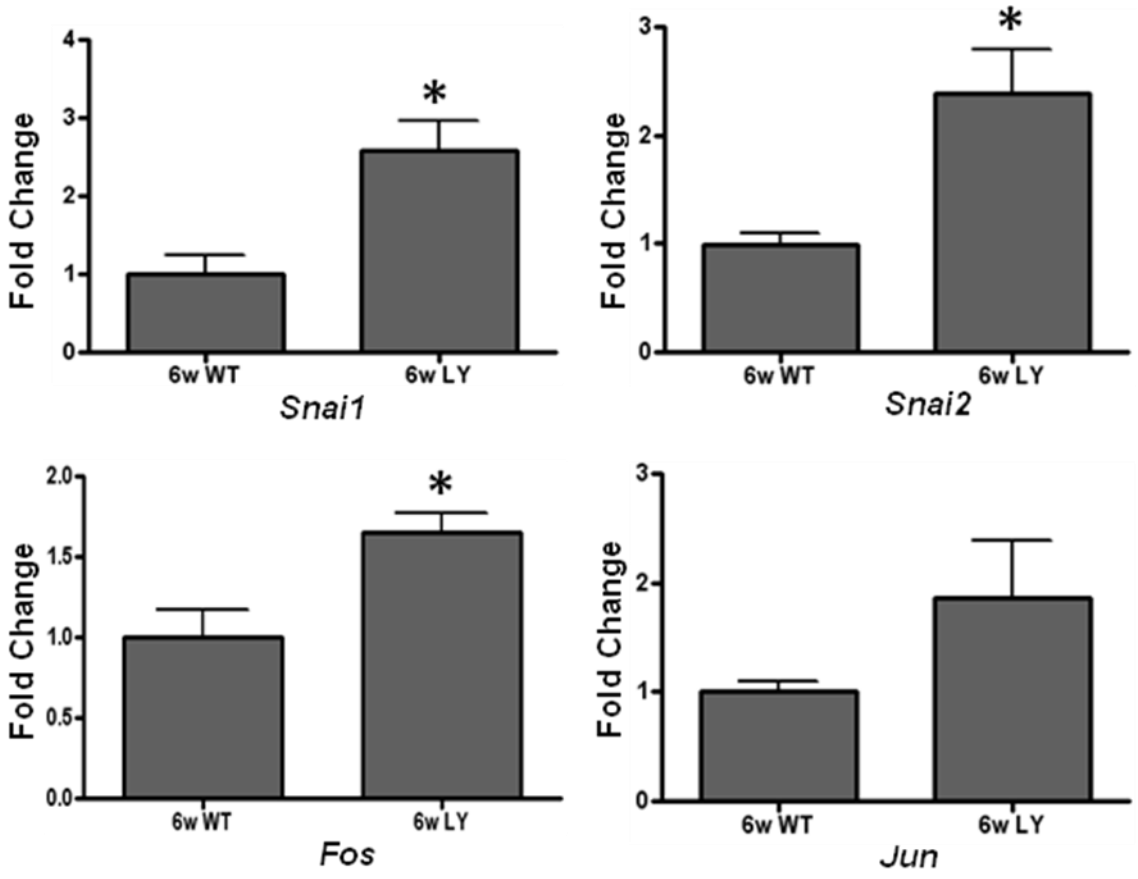

Figure 2.1. **IGF-1 dependent regulation of IE and EMT genes expression in mouse uterus.** Uterus tissue were isolated from B6 (n = 5) and LY (n = 5) 6 week mice. Isolated the RNA and QPCR was carried out using primers against *Snai1, Snai2, Jun* and *Fos*. The mRNA abundance of each candidate in each sample was normalized by GAPDH mRNA abundance and expressed as a fold change. Candidate gene expression from the Uterus of LY at 6 weeks was subsequently compared to B6 mice. All QPCR data was tested for significant differences in mRNA abundance using t-test. Alphabet letters show significant fold change ($P < 0.05$). *Error bars* represent mean ± SE.

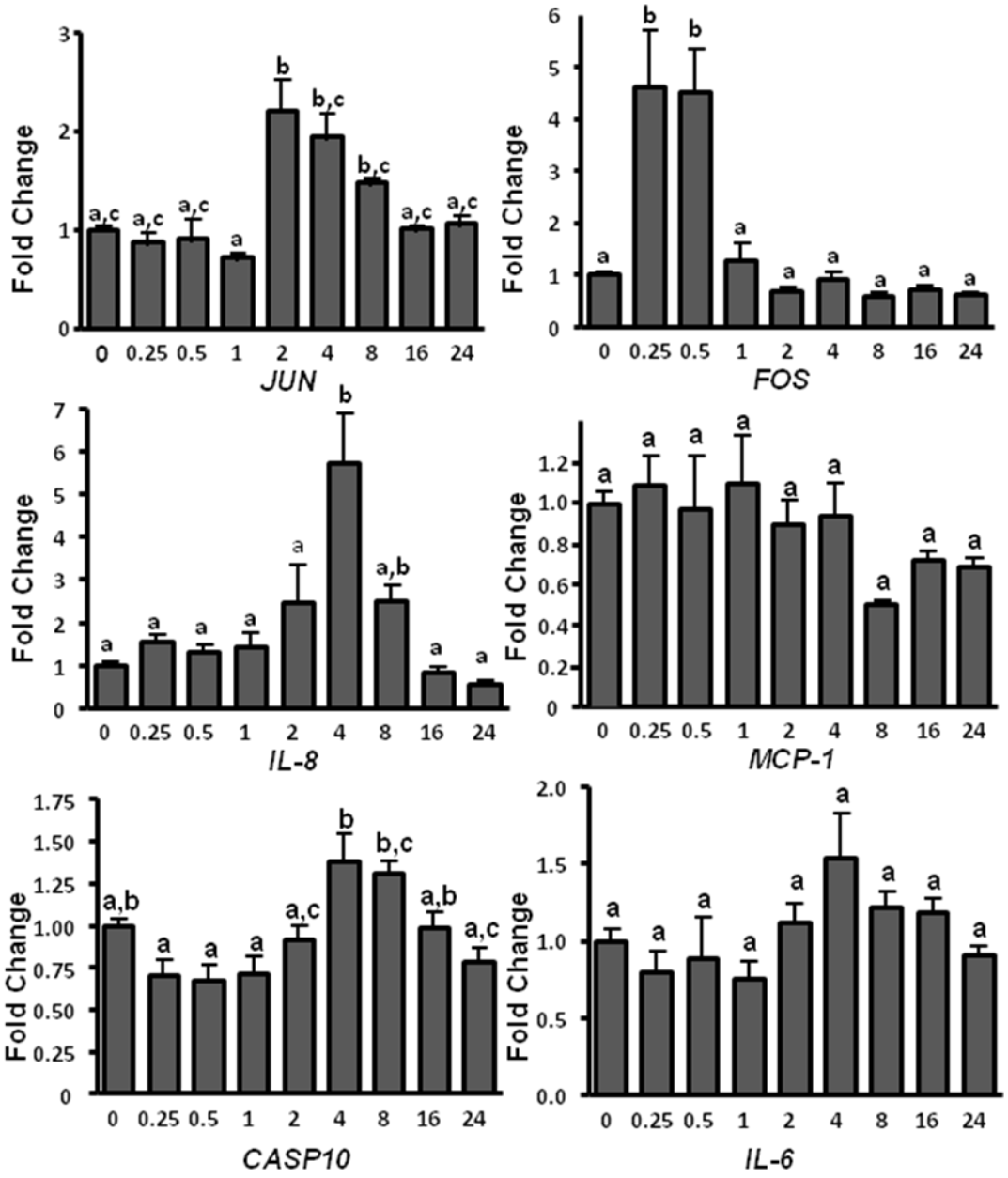

Figure 2.2. **IGF-1 dependent regulation of immediate early genes expression of HeLa cells.** 200,000 HeLa cells were cultured in DMEM maintenance media in 3.5 cm dish. After 24 h serum starvation, cells were treated with 100 ng/ml IGF-1 for 0, 0.25, 0.5, 1, 2, 4, 8, 16 or 24 h. Cells were collected. Isolated the RNA and QPCR was carried out using primers against *JUN, FOS, IL-8, IL-6* and *CASP10*. The mRNA abundance of each candidate in each sample was normalized by GAPDH mRNA abundanc and expressed as a fold change. Comparing the candidate gene expression in different time points. All QPCR data was tested for significant differences in mRNA abundance using one-way ANOVA. Alphabet letters show significant fold change ($P < 0.05$). *Error bars* represent mean ± SE.

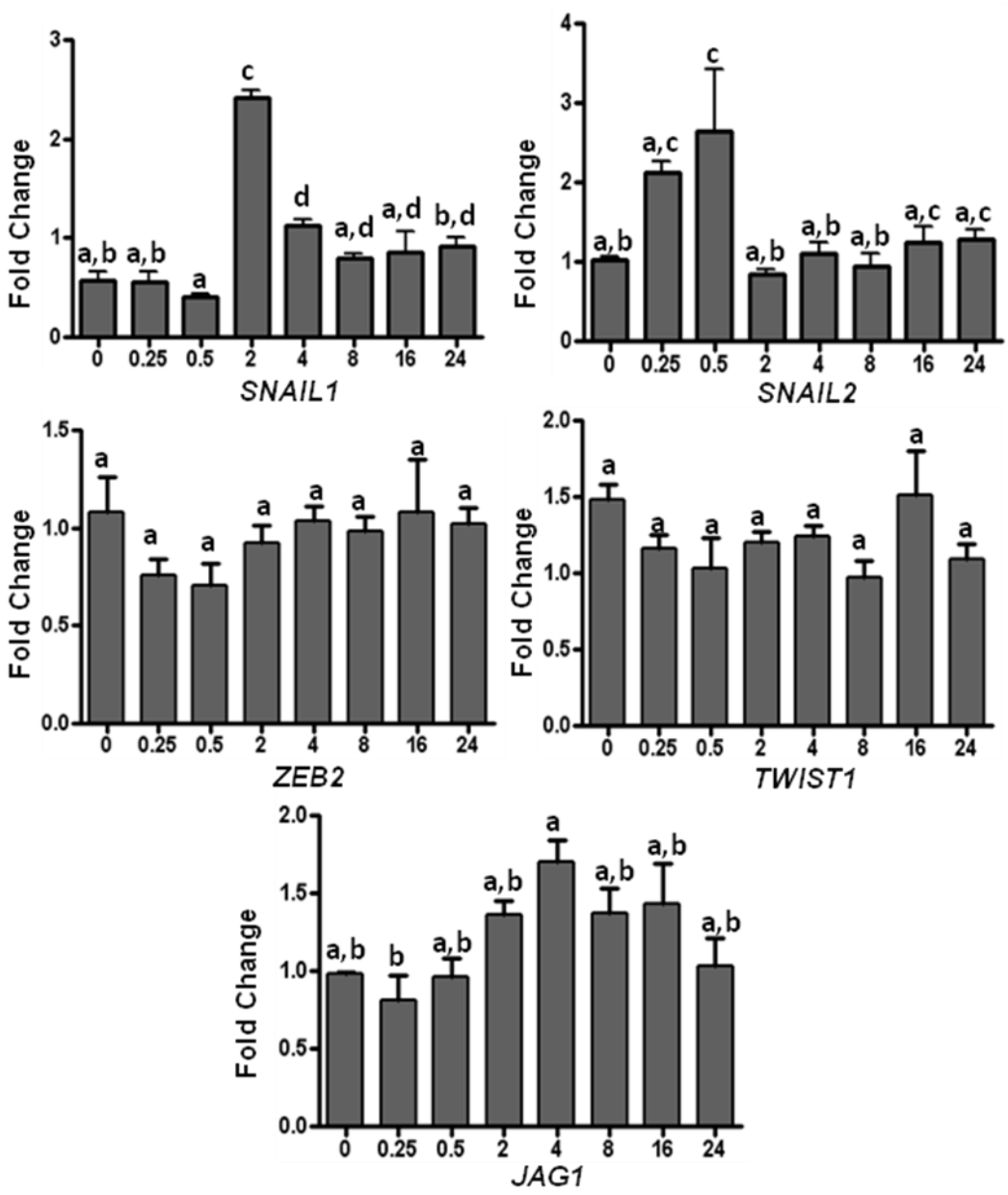

***Figure 2.3*.** **IGF-1 dependent regulation of epithelial transition markers genes expression of HeLa cells.** 200,000 HeLa cells were cultured in DMEM maintenance media in 3.5 cm dish. After 24 h serum starvation, cells were treated with 100 ng/ml IGF-1 for 0, 0.25, 0.5, 1, 2, 4, 8, 16 or 24 h. Cells were collected. Isolated the RNA and QPCR was carried out using primers against *SNAI1, SNAI2, TWIST, CDH1, ZEB2,* and *JAG1*. The mRNA abundance of each candidate in each sample was normalized by GAPDH mRNA abundance and expressed as a fold change. Comparing the candidate gene expression in different time points. All QPCR data was tested for significant differences in mRNA abundance using one-way ANOVA. Alphabet letters show significant fold change ($P < 0.05$). *Error bars* represent mean ± SE.

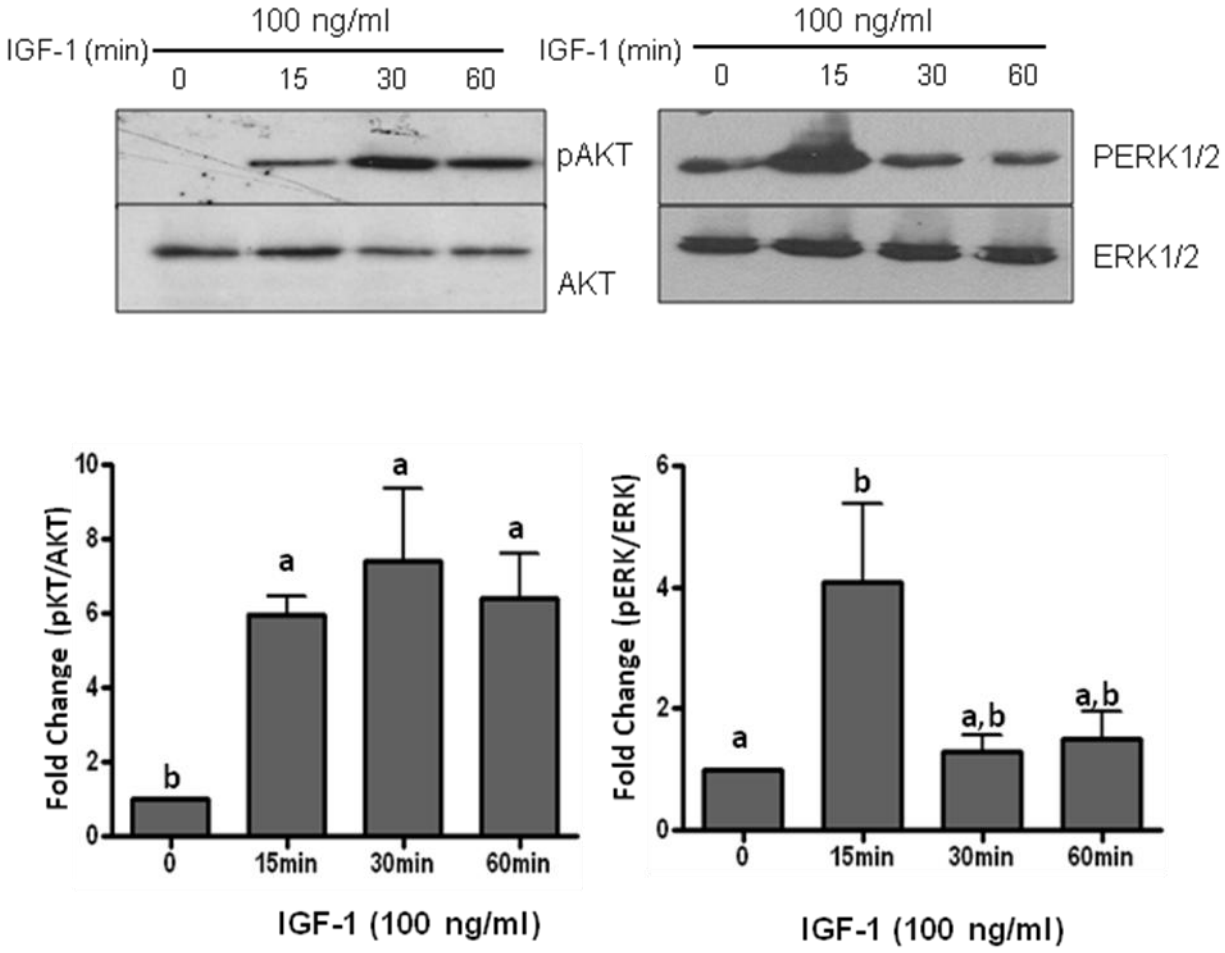

Figure 2.4. **IGF-1 activation of AKT phosphorylation and ERK1/2 phosphorylation**. 500,000 HeLa cells were cultured in DMEM maintenance media in 10 cm dish. After 24 h serum starvation, cells were treated with 100 ng/ml IGF-1 and then protein extracts were collected from HeLa cells treated with IGF-1 for 0, 15, 30 or 60 minutes. Western blot analysis was carried out using antibodies against phosphorylated pAKT or phosphorylated pERK1/2. Total AKT or total ERK1/2 was subsequently probed and served as loading controls. Semi-quantitative analysis of band density was calculated for statistic analyses using one-way ANOVA. Different alphabet letters show significant differences ($P < 0.05$) of phospho-protein/total protein ratio in time course. The *Error bars* represent mean ± SE.

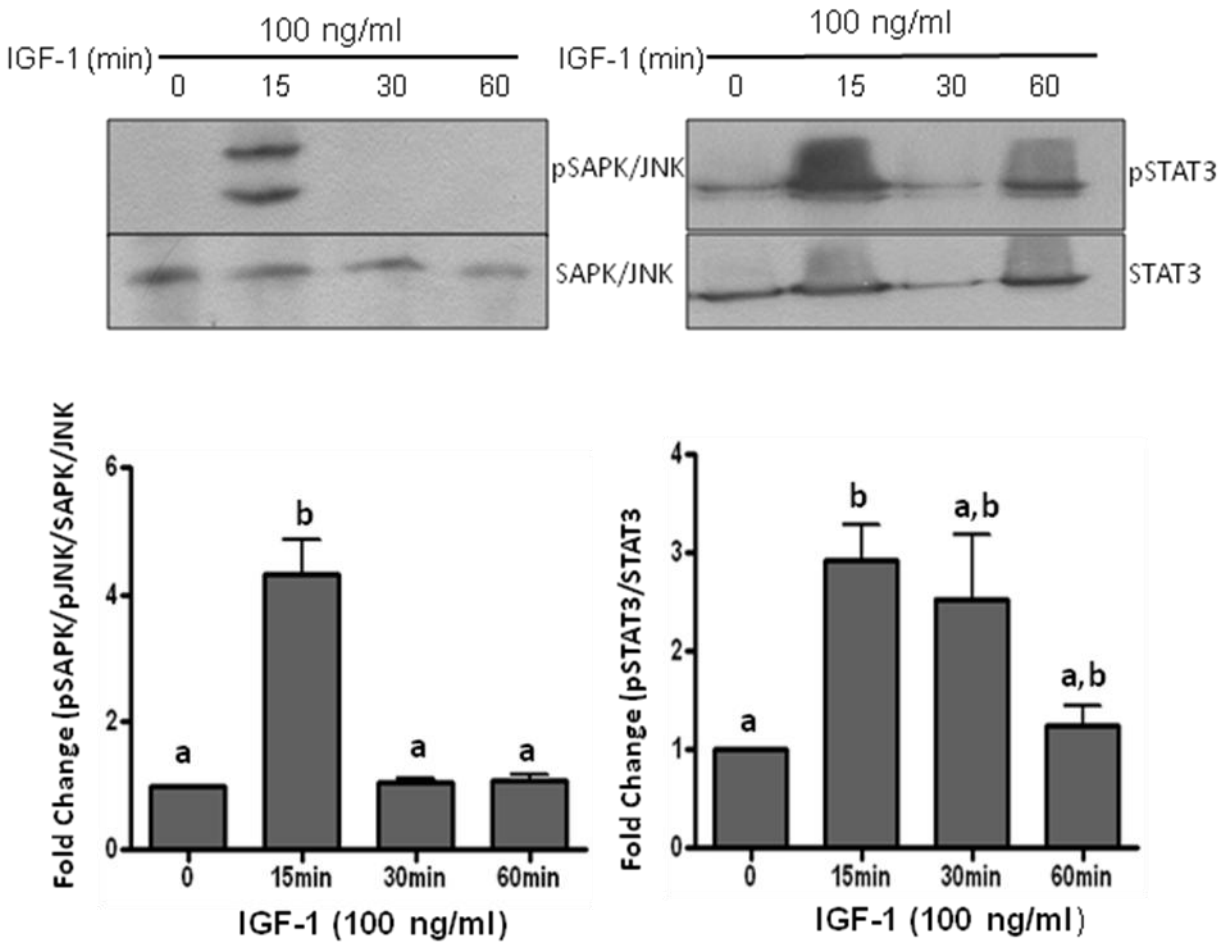

Figure 2.5. **IGF-1 activation of SAPK/JNK phosphorylation and STAT3 phosphorylation.** 500,000 HeLa cells were cultured in DMEM maintenance media in 10 cm dish. After 24 h serum starvation, cells were treated with 100 ng/ml IGF-1 and then protein extracts were collected from HeLa cells treated with IGF-1 for 0, 15, 30 or 60 minutes. Western blot analysis was carried out using antibodies against phosphorylated pSAPK/JNK or phosphorylated pSTAT3. Total SAPK/JNK or total STAT3 was subsequently probed and served as loading control. Semi-quantitative analysis of band density was calculated for statistic analyses using one-way ANOVA. Different alphabet letters show significant differences ($P < 0.05$) of phospho-protein/total protein ratio in time course. The *Error bars* represent mean ± SE.

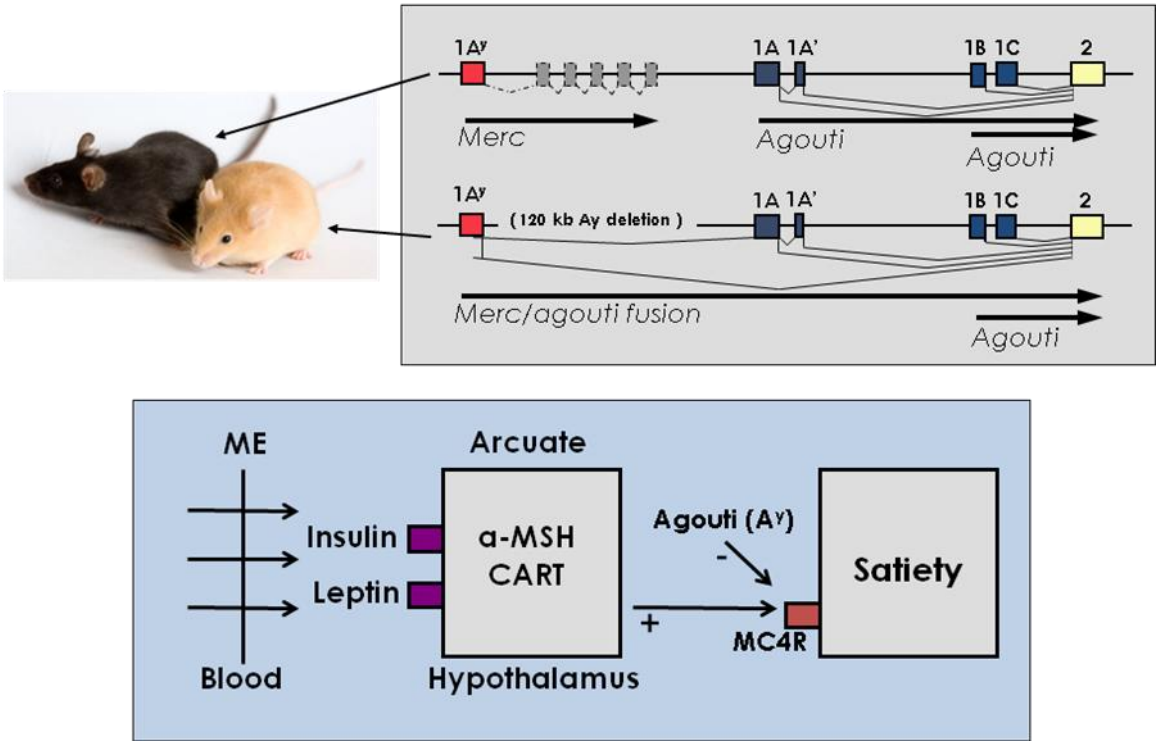

Figure 2.6. Lethal yellow mouse model. The LY mouse own a gene deletion in the promoter and first exon region of the agouti gene which locus on the C57BL/6 background. The deletion causes the ubiquitous expression of the agouti gene. Over-expressed agouti acts as an antagonist of a-MSH by binding to melanocortin-4 receptors (MCR4) in the hypothalamus. Therefore, over-expressed agouti protein interfers with normal satiety control. As a consequence, the LY mice overeat resulting in the development of obesity.

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CHAPTER 3

Regulation of Immediate Early and EMT Markers by leptin in HeLa Cells

Abstract

Obesity increases the risk factor in various cancers including breast, endometrial, ovarian, prostate cancer esophagus, gastric, colon cancer, leukemias. Leptin acts as significant role in the pathophysiology of obesity. Given that, the increased leptin level in obese individuals, the study suggests the relationship of leptin's link to cancer risk is of such considerable importance. In the current study, after 24h serum starvation, HeLa cells were treated with 100ng/ml leptin for 0, 0.5,1,2,4 or 8 hours. After treatment, RNA was collected and quantitative PCR (QPCR) was carried out using primers against immediate early (IE) genes JUN, FOS, IL-8, IL-6, MCP-1 and CASP10 and epithelial-mesenchymal transition (EMT) marker genes SNAI1, SNAI2, Twist, CDH1, ZEB2. Leptin stimulated a significant transient increase in JUN, IL-6 and MCP-1 with the peak showing at 8 hour. The signaling pathways including ERK1/2, STAT3, and SAPK/JNK is important for the regulation of the IE and EMT. Western blot analyses demonstrated increased phosphorylation of STAT3, and SAPK/JNK by leptin in HeLa cells. In all, this study has identified a novel mechanism for important relationship between leptin and cancer in obesity. It may be another key to understand cancer and provide new methods to care cancer.

Introduction

Obesity is a prevalent condition which is often stigmatized. Nearly two-thirds of the United States population is overweight. Since 1980, more than one-third of the population are at least 20% above their normal weight in the USA, and this proportion is increasing (3, 4). Increasingly, obesity has become a serious medical problem in developing nations with urbanization and a more plentiful food supply. However, obesity is not just meaning too much fat, in fact, it reduce life expectancy is reduced when body-mass index (BMI, body mass index) is 20% or more above normal (1). Furthermore, obesity is followed with the increased likelihood of various diseases, including heart disease, type 2 diabetes, high blood pressure, weight-bearing arthritis , asthma and certain types of cancers (2).

In individuals, energy balance is normally controlled by a feedback loop which interacts with the satiety center in the brain, which maintains constancy of total body energy stores. In the obese phenotype, there is an increase in plasma leptin suggesting that obesity results in resistance to leptin and disruption of satiety control which further exacerbates the obesity. In recent years, leptin has become a focus for obesity-dependent diseases because the key role to balance energy intake.

Leptin is from the Greek word "leptos," meaning thin. It is a peptide hormone of 16kDa and 167 amino acids; one of the important adipose derived hormones which regulates energy intake and expenditure. Leptin is the product of the "obesity" (ob) gene which is discovered in 1994 by Zhang (5). In an individual, leptin is expressed primarily by the adipocytes of white adipose tissue and, at lower levels, in gastric epithelium and placenta (6, 7). In the central nervous system, leptin interacts with the complex pathways

and also through direct peripheral mechanisms. Leptin acts as a mediator of energy expenditure (8); as a permissive factor to mediate puberty (9); as a signal of metabolic status during pregnancy to modulate the fetus and the maternal metabolism (10); and as a mediator to interact with other hormones to regulate the energy status and metabolism including insulin, the insulin-like growth factors, growth hormone and glucocorticoids (11-13). Leptin was considered as a great hope to be the successful treatment of obesity for scientific and clinical communities. But it is just a dream to use leptin to treat obesity. The complex leptin axis and the existence of leptin resistance in most obesity make the dream gone with wind. However the role of the leptin axis is now being shown to have more functions than it was first discovered. Complex interactions between hormone axes in the periphery are integral in maintaining homeostasis of a diverse range of functions.

Though obesity is recognized as an established risk factor in various cancers; it is only recently that studies have established a link between leptin and cancer. For example, leptin was reported to play an important role in serious human reproduction cancers such as breast, endometrial, ovarian and prostate cancer (14-17). Leptin promotes the growth of malignant cells by activating signal transducer and activator of transcription 3 (STAT3) and kinase (ERK) 1/2 pathways in breast cancer (18). Leptin might be a potential regulator for ovarian cancer, as leptin receptors were identified in ovarian surface epithelium and ovarian cancer cell lines (16). Obesity, a situation characterized by high levels leptin, has also been examined as a potential increased risk factor for prostate cancer but there is no clear evidence to support a strong pathogenic link between obesity and prostate cancer (17, 19). However, leptin may cause the alterations of the balance of sexual hormones, which result in increased risk of prostate cancer (17, 19). In

all, leptin could be one of the important key to determine the reason of the increased risk of cancer in obesity.

MATERIALS AND METHODS

HeLa cell culture and whole cell protein extracts: 500,000 HeLa cells (American Type Culture Collection, Manassas, VA) were cultured in 10 cm dish with Eagle's Minimum Essential Medium pH 7.4 (Sigma, St. Louis, MO), containing 1.5 g/L NaHCO3, 0.11 g/L Sodium pyruvate, 0.292 g/L L-glutamine, 10 ml/L Penn-strep, 1 ml/L phenol red and 100 ml/L of heat inactivated FBS (Hyclone, Logan, UT, USA). After 24h, the cells were changed to the same medium except there was no FBS included for serum starvation. After 24h serum starvation, cells were treated with 100ng/ml leptin (Sigma, St. Louis, MO) for 0, 15, 30 or 60 minutes. The cells were washed with 1X HBSS (Invitrogen, Carlsbad, CA, USA) and cells collected in RIPA buffer (150 mM NaCl, 1 mM EDTA, 50mM Tris-HCl pH7.4, 1% NP-40, 0.25% Na-deoxycholate) containing phosphatase inhibitors (1 mM NaF and 1 mM Na3VO4) and Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics). Cells in the RIPA buffer were sonicated 15 seconds and then centrifuged at 10,000 x g for 5 minute to separate cell debris and the supernatant which contain soluble protein. The protein concentration of each experimental replicate was determined with the Pierce BCA Protein Assay (Rockford, IL, USA).

HeLa cell culture and RNA extraction: 200,000 HeLa cells (American Type Culture Collection, Manassas, VA) were cultured in 3.5 cm dish and serum starved for 24 hours as described above. After serum starvation, cells were treated with 100ng/ml leptin

(Sigma, St. Louis, MO) for 0, 0.25, 0.5, 1, 2, 4, or 8 hours. The cells were washed with 1X HBSS (Invitrogen, Carlsbad, CA, USA) and then collected in TRI reagent (Ambion Inc., Austin, TX, USA). Total RNA was extracted according to the manufacturer's protocol (Sigma, St. Louis, MO). After isolation, the total RNA was dissolved in 20 µl diethyl pyrocarbonate (DEPC) water. The RNA concentration was determined using Beckman Coulter DU 730 Life Science UV/Vis Spectrophotometer.

Reverse Transcription: Total HeLa cell RNA (5 µg) was mixed with 5 units of RQ1 RNase free DNase (Promega, Madison, WI), M-MLV RT buffer and DEPC water and incubated at 37°C for 30 minutes to remove genomic DNA contaminants. Before RT-PCR, 1.1 µl of RNA and RQ1 mix was removed from each sample to a new tube with 15 µl DEPC water, which should be used as No RT to detect the residual genomic DNA. The mixed RNA samples was subsequently combined with 2 µl Random primers (Promega), 2 µl dexoxynucleotide triphosphates (Fermantas; 10 mM dNTP mix), 2.4 µl DEPC water, and then were incubated in 65°C for 5 min. After that, the mixed samples were chilled on the ice quickly. 400 units of Moloney Murine Leukemia Virus reverse transcriptase (Promega), 3.2 µl of RT buffer (Invitrogen, Carlsbad, CA), and 2 µl 0.1 M DL-dithiothreitol (DTT) (Invitrogen, Carlsbad, CA) were added into each sample. The mixed samples were incubated at 37°C for 2 hour and followed by 15 min at 72°C to stop the reverse transcriptase. The cDNA was stored at -20°C for subsequent real time-PCR.

Quantitative, Real-Time PCR (qPCR) Analysis: Gene-specific forward and reverse primers were designed (Primer Express, Applied Biosystems, Foster City, CA) and synthesized (Integrated DNA Technologies, Coralville, IA) and store at -20°C. The information of the primers and probes are shown in table 3.1. Each set of gene-specific

primers was tested to determine the maximal concentration of primers that could be used to produce specific amplification of the target sequence without primer dimer amplification. Before use, each cDNA sample was diluted 1:10. Glyceradehyde-3-phosphate dehydrogenase (Gapdh) gene is a housekeeping gene and used as a control for reverse transcription efficiency and well-to-well variation in template. PCR was performed with 10 µl Taqman Universal Master Mix (Applied Biosystem, Foster City, CA), 1 µl cDNA, 1 µl probe with primers, and 8 µl DEPC to make up the reaction volume to 20 µl. QRT-PCR was performed in 364 well plates (Axygen Scientific, Union City, CA) with an adhesive cover film (VWR, Scientific Products, North Kansas City, MO) in 7900HT Fast Real-Time PCR system (Applied Biosystems). Quantitative PCR (qPCR) reactions of the other target genes were carried out using 1:10 dilutions of each cDNA sample and standard samples with Power SYBR Green PCR Master Mix (Applied Biosystem, Foster City, CA). All experimental and Gapdh PCRs were carried out in separate wells in triplicate. These relative values are plotted against the threshold value for each dilution to generate a standard curve. An arbitrary value of template was assigned to the highest standard and corresponding values were assigned to the subsequent dilutions. According to the slope and y-intercept of the standard curve, 7900HT Fast Real-Time PCR system assigned a value of the relative amount for each experimental and Gapdh triplicate. The value of the average of the experimental triplicate divided by the average of the Gapdh triplicate was used for statistical analysis.

Western Blot Analyses: The protein samples were resolved by SDS-polyacrylamide 10% gel electrophoresis which contains 4% stacking gel and 10% separating gel. Protein samples were loaded together with loading buffer and the

separated protein was transferred to Immobilon PVDF (Millipore, Billerica, MA). Following transfer, the membranes were blocked with 5% nonfat dry milk in 1X TBST (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 01% (v/v) Tween 20) for 1 hour with gentle shaking in order to block the nonspecific proteins. After that, the blots were probed with the primary antibodies which are diluted in 5% w/v BSA, 1XTBS, 0.1% Tween 20. The membrane was then incubated with primary antibody against phospho-AKT (Cell Signaling Technology, Danvers, MA), phospho-ERK1/2 (Cell Signaling Technology), phospho-SAPK/JNK (Cell Signaling Technology), or phospho-STAT3 (Cell Signaling Technology) overnight at 4°C with gentle shaking. Blots were washed with 1X TBST and incubated for 1 hour with HRP-conjugated secondary antibody which was diluted with 5% nonfat dry milk in 1X TBST (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 01% (v/v) Tween 20). Proteins were incubated with West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) for 5 min and exposed to autoradiograph film (Fisher Scientific, Fairlawn, IL) in dark room. After the visualization of phosphorylated protein, the blots were stripped with Restore plus Western Blot Stripping Buffer (Pierce) in room temperature for 20 min and 37°C 10 min. Wash the blots with 1X TBST and blocked with 5% milk in 1XTBST. Next, incubated with primary antibody against total AKT, total ERK1/2, total SAPK/JNK, or total STAT3 (Cell Signaling Technology) overnight at 4°C with gentle shaking. Total protein was exposed and visualized as described above. The visualized total protein served as a loading control for each sample. The autoradiograph images films were scanned and the density of the protein band was determined in Photoshop. Semi-quantitative analysis of band density of the phosphorylated and total protein was calculated in each film as described by Miller

(http://lukemiller.org/journal/2007/08/quantifying-western-blots-without.html). The amount of phosphorylated protein was normalized by total protein expressed in each sample. The normalized abundance of phosphorylated protein of each sample was subsequently compared to the untreated control sample. The data was showed as a fold-change.

Statistical Analyses: All statistical analyses were carried out by GraphPad Prism 4.0 (Graphpad Software, La Jolla, CA). QPCR data and western-blot data were analyzed using one-way ANOVA. Differences in data were considered statistically significant at $P < 0.05$. Different letters indicate significant differences in fold change in mRNA abundance compared to control for all qPCR data and phospho-protein/total protein ratio in time course. The Error bars represent mean ± SE.

Results

Significant gene expression differences In HeLa cells by Leptin

To evaluate effects of leptin on the immediate early gene expression, HeLa cells were treated with 100 ng/ml leptin after 24-hour serum starvation. The abundance of each mRNA transcript was analyzed by quantitative real-time PCR (qPCR). These analyses indicated that *JUN* ,*FOS* , *IL-8* and *CASP10* are modestly increased 0.5 hours after leptin treatment, although statistically insignificant, as compared to control. MCP-1 and IL-6 began to show a trend of increased mRNA at 0.5 hour. At 8 hours, their mRNA increased significantly by 1.5- and 2-fold, respectively compared to untreated control cells (Fig 3.1). Conversely, 100 ng/ml of leptin had no effect on mRNA level of epithelial-mesenchymal transition marker gene expression, including SNAI1, SNAI2, Twist, ZEB2, and JAG1. There is no significant difference in mRNA abundance

compared to untreated control cells at any time point (Fig. 3.2).

Signaling Pathways regulated by leptin in HeLa cells

ERK1/2 and JNK-STAT pathways are important mediators of leptin effects on cell function. In Fig 3.3, 100 ng/ml of leptin only had a modest effect on the phosphorylation of ERK1/2 over the time course. Specifically, phosphorylated ERK1/2 was increased by 1.5 fold in 15 minutes. In Fig 3.4, leptin increased SAPK/JNK and STAT3 phosphorylation by 1.5- or 2- fold, respectively as early as 15 minutes after treatment. After that, SAPK/JNK phosphorylation began to decrease toward untreated control levels, but increased STAT3 phosphorylation level was maintained over the entire time course.

Discussion

Obesity, characterized by high levels of leptin, has also been examined as a potential increased risk factor for cancer but there is no clear evidence to support a strong pathogenic link between leptin and cancer. However, leptin cause the alterations of the balance of sexual hormones, which result in increased risk of prostate cancer (17, 19). Leptin was reported to play an important role in a serious human reproduction cancer such as breast, endometrial, ovarian and prostate cancer (14-17). Recently, more and more attentions were focused on unbalance hormone level in obesity, such as leptin. The leptin levels, which play an important role in obesity , were compared between LY and B6 males and females (20, 21). According to the previous data of our lab, leptin levels were significantly increased in LY female mice compared to age-matched controls at both 12 and 24 weeks of age.

To determine potential mechanisms for obesity-induced cancer, HeLa cells were treated with leptin in this study. Our data demonstrated that leptin increased the expression of inflammation genes MCP-1 and IL-6 at 8 hours. It was reported previously that leptin induced the expression of *IL-6* and *MCP-1* in obese mice and lean Wistar Rats (22, 23). Our study similarly showed leptin-dependent increase in IL-6 and MCP-1 expression although the induction magnitude was not as great and likely reflects differences between chronic and acute exposure to leptin. In addition, advanced/metastatic cancer patients have higher levels of IL-6 in their blood (24). There is an elevated levels of IL-6 in murine models of colitis-associated cancer (CAC) (25) and the higher serum levels of IL-6 exhibited in males could be the reason of gender bias in liver cancer susceptibility (26). IL-6 promotes the in vivo growth of tumors in models of prostate, breast, and lung cancers (27). The increased IL-6 may be the important link between increased leptin and obesity related cancer. In fact, obesity is associated with a chronic inflammatory response characterized by over expressed cytokine such as IL-6 , TNFα, inhibitor of NF-κB kinase b (IKKb), macrophage migration inhibitory factor, nerve growth factor, vascular endothelial growth factor, plasminogen activator inhibitor 1 and haptoglobin; increased acute-phase reactants, and activation of inflammatory signaling pathways (28, 29).

Increased IL-6 and MCP-1 play very important roles in obese mice. Interestingly, IL-6 has pro-inflammatory and anti-inflammatory function. IL-6 is secreted by immune cells including T cells and macrophages in response to foreign pathogen stimulus, in order to activate inflammation (22, 23). Thus, the increased leptin may play a role to induce the micro-inflammatory condition in obesity. In fact, adipocytes are able to

produce IL-6, as reported that endogenous CRP level is very high in obese individuals (30). The inflammatory conditions in obesity may initiate or promote oncogenic transformation, and also can generate an inflammatory microenvironment that promotes tumor progression. MCP-1, is a small cytokine that functions as recruitment of monocytes, memory T cells, and dendritic cells to sites of tissue injury, infection, and inflammation. MCP-1 acts as chemotactic activity for monocytes and basophils but not for neutrophils or eosinophils (31). Interestingly, many tumors are reported to produce abundant MCP-1, including melanoma (32), ovarian cancer (33), and breast carcinoma (34). Activation of MCP-1/CCR2 axis promotes prostate cancer growth in bone.

In the current study, leptin did not affect FOS/JUN expression in 4 hours. While in 8 hours, JUN mRNA appeared to be up-regulated. Jun, in combination with c-Fos, is required for the increased activity at AP-1 promoters. AP-1 activity depends on both double phosphorylations by the JNK pathway, and phosphorylation-independent pathway. AP-1 proteins participate in tumorigenesis by regulating oncogenic transformation, proliferation, apoptosis, invasive growth and angiogenesis (35). c-Jun protein was established as an oncogene at the early stage of hepatocellular carcinoma in a mouse mode (36). In mice with c-Jun deletion, the number and size of hepatic tumors were reduced. This process was related to increased p53 level and apoptosis (36). In mouse development and tumorigenesis model by Jochum's, c-Jun binds to the promoter of the P53 and cyclin D1, and then inhibit the p53 gene expression and stimulate the cycline D1 gene expression (37). Inhibition of AP-1 activity by over-expressing a dominant negative form of cJun (cjun-DN, Tam67) inhibits breast cancer cell growth. Targeting the AP-1 transcription factor may be a key for the treatment of breast cancer

(38, 39). Our data indicated leptin was able to increase JUN mRNA level in HeLa cells. This suggests that JUN inhibition provides a promising therapeutic method for obesity related cancer. However, we didn't find leptin was able to regulate the expression of epithelial transition marker genes, including SNAI1/2, ZEB2, TWIST1, or JAG1 at early hours in HeLa cells.

To seek for the early mediators which deliver leptin signaling, we demonstrated that leptin was able to activate ERK1/2, JNK-STAT early at 15 minutes. Some studies showed that leptin increased cancer cell growth through the ERK1/2 and JNK-STAT pathway, increased cancer cell invasion via a PI-3K. In addition, the nuclear expressions of ERK1/2 might be useful markers for tumor invasiveness and lymph node metastasis in stomach cancer. Leptin promotes the growth of malignant cells by activating signal transducer and activator of transcription 3 (STAT3) and kinase (ERK) 1/2 pathways in breast cancer (46). ERK pathway is activated by a variety of stimuli including growth factors, cytokines, virus infection, and carcinogens. It has been reported that leptin markedly activated ERKs and JNK. In contrast, these stimulatory effects of leptin on ERKs and JNK were completely inhibited by a PKC inhibitor Ro 32-0432 as well as EDTA. In this process, Ca2+-independent novel PKC provides a link between leptin and ERKs/JNK (47).These results provide an appropriate explanation on our finding.

ERKs pathway activates Fos and Jun, resulting in activation of AP-1. The transcriptional activity of c-Fos is regulated by phosphorylation at Ser374 by ERK. JNK also activates C-FOS via activation of ERK-1. JNK phosphorylates c-Jun at Ser63 and Ser^{73}(48, 49). STAT signaling is essential for the regulation of food intake and energy expenditure by leptin. Binding of leptin to its receptor stimulates gene transcription via

activation of STAT proteins. In Ishikawa cells, leptin was reported to increase STAT3-DNA binding activity to Cyclin D1 promoter, enhance recruitment of STAT3 to the promoter region of cyclin D1 (50). In contrast, STAT3 knockdown prevented leptin-induced cyclin D1 expression and cell proliferation (50). Our data show the increased phosphorylation of STAT3 though it is not significant. All of these data suggested a strong link between leptin and tumor progression. However, the precise network between leptin and ERK, JNK, and STAT pathway activation remain unclear. Further studies will be needed to clarify these questions.

In summary, our data also indicated that both leptin and IL-6/MCP-1 activated STAT3. Therefore, IL-6/MCP-1-STAT3 axis is a potential important pathway in obesity-dependent cancers. In addition, there is an interest in developing anti-IL-6/MCP-1 agents as therapy against obesity and cancers.

Table 3.1. Human Primer sequences used for QPCR analysis

Gene	**Probe Dye**	**Primer**	**Sequence (**5' to 3'**)**
CASP10	SYBR Green	Forward	GGAGCTGTCTACTCTTCGGATGA
		Reverse	AGGGCTGTGAAGTGAGACATGAT
FOS	SYBR Green	Forward	CCTCGCCCGGCTTTG
		Reverse	GCCTCGTAGTCTGCGTTGAAG
JUN	SYBR Green	Forward	CTGGGAGGACCGGAGACA
		Reverse	GAGAAGCCTAAGACGCAGGAAA
MCP-1	SYBR Green	Forward	CAAGCAGAAGTGGGTTCAGGAT
		Reverse	TCTTCGGAGTTTGGGTTTGC
IL-6	SYBR Green	Forward	AGGGCTCTTCGGCAAATGTA
		Reverse	GAAGGAATGCCCATTAACAACAA
IL-8	SYBR Green	Forward	CTGGCCGTGGCTCTCTTG
		Reverse	CCTTGGCAAAACTGCACCTT
SNAI1	SYBR Green	Forward	CCCCAATCGGAAGCCTAACT
		Reverse	GCTGGAAGGTAAACTCTGGATTAGA
SNAI2	SYBR Green	Forward	CCTGGGCGCCCTGAA
		Reverse	TTCTCCCCCGTGTGAGTTCT
TWIST1	SYBR Green	Forward	TCCGCGTCCCACTAGCA
		Reverse	AGTTATCCAGCTCCAGAGTCTCTAGAC
ZEB2	SYBR Green	Forward	CCAGCTCGAGCGGCATA
		Reverse	GCCACACTCTGTGCATTTGAA
JAG1	SYBR Green	Forward	CAGCTCTGTGACAAAGATCTCAATTAC
		Reverse	AGGGCCTGTGTTGCTACAAGTT
GAPDH	Kit-Taqman Rodent GapDH Control Reagents Applied Biosystems (VIC Probe)		

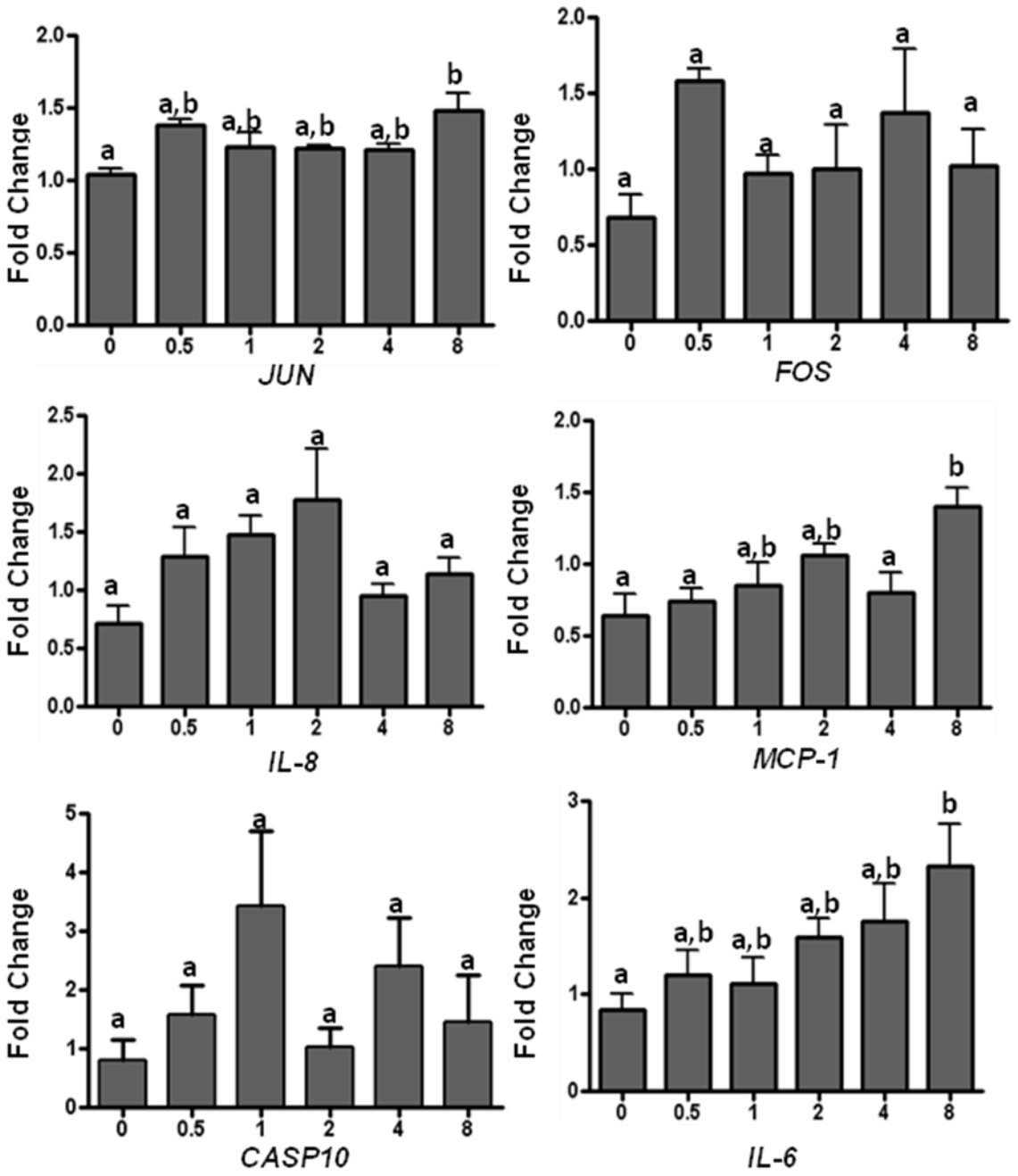

Figure 3.1. **Leptin dependent regulation of immediate early genes expression of HeLa cells.** 200,000 HeLa cells were cultured in DMEM maintenance media in 3.5 cm dish. After 24 h serum starvation, cells were treated with 100 ng/ml leptin for 0, 0.5, 1, 2, 4 or 8 h. Cells were collected. Isolated the RNA and QPCR was carried out using primers against *JUN, FOS, IL-8, IL-6* and *CASP10*. The mRNA abundance of each candidate in each sample was normalized by GAPDH mRNA abundance and expressed as a fold change. Comparing the candidate gene expression in different time points. All QPCR data was tested for significant differences in mRNA abundance using one-way ANOVA. Alphabet letters show significant fold change ($P < 0.05$). *Error bars* represent mean ± SE.

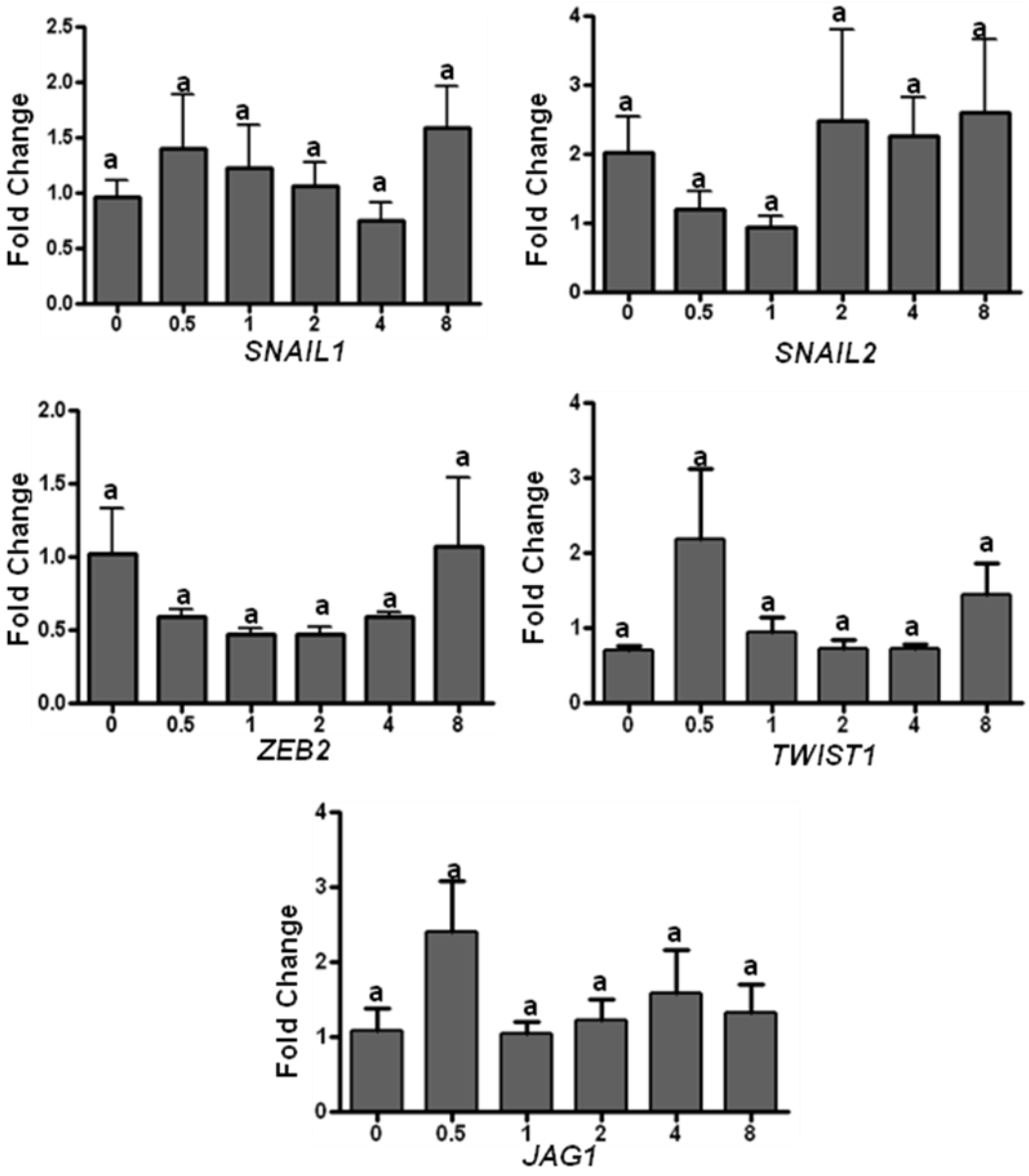

Figure 3.2. **Leptin dependent regulation of epithelial transition markers genes expression of HeLa cells.** 200,000 HeLa cells were cultured in DMEM maintenance media in 3.5 cm dish. After 24 h serum starvation, cells were treated with 100 ng/ml leptin for 0, 0.5, 1, 2, 4, or 8 h. Cells were collected. Isolated the RNA and QPCR was carried out using primers against *SNAI1, SNAI2, TWIST1, CDH1, ZEB2,* and *JAG1*. The mRNA abundance of each candidate in each sample was normalized by GAPDH mRNA abundance and expressed as a fold change. Comparing the candidate gene expression in different time points. All QPCR data was tested for significant differences in mRNA abundance using one-way ANOVA. Alphabet letters show significant fold change ($P < 0.05$). *Error bars* represent mean ± SE.

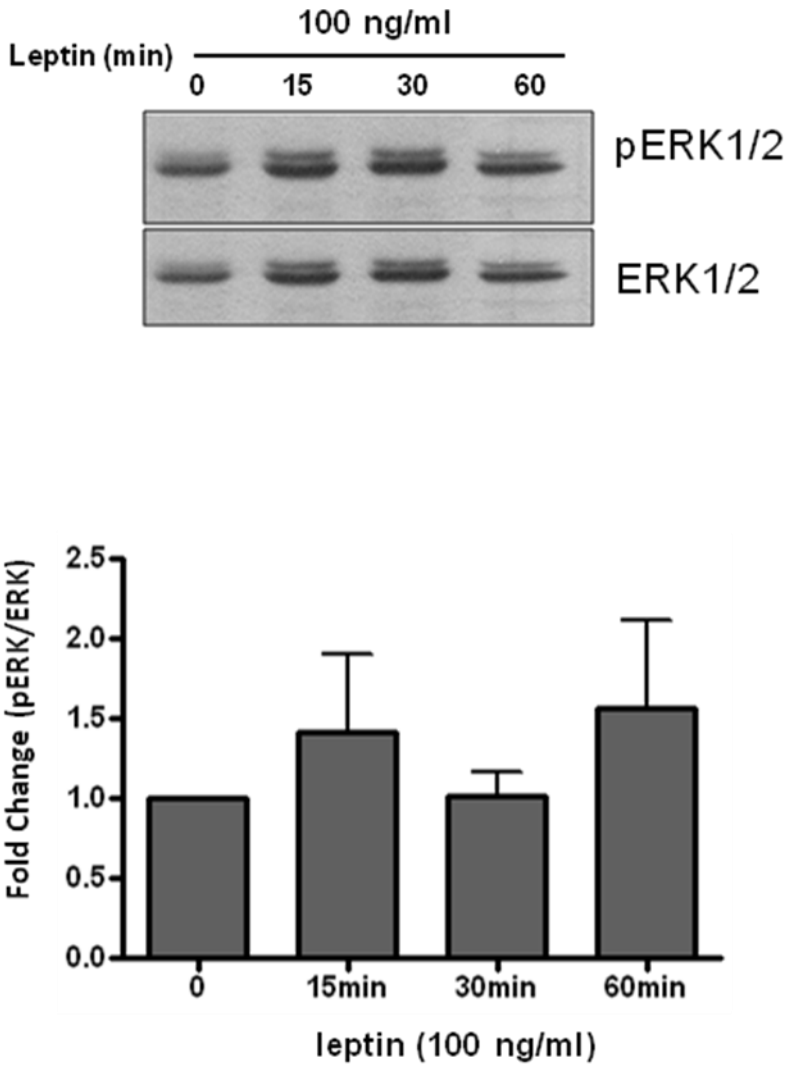

Figure 3.3. **Leptin activation of Erk1/2 phosphorylation.** HeLa cells were cultured in DMEM maintenance media in 10 cm dish. After 24 h serum starvation, cells were treated with 100 ng/ml Leptin and then protein extracts were collected from HeLa cells treated with Leptin for 0, 15, 30 or 60 minutes. Western blot analysis was carried out using antibodies against phosphorylated pERK1/2. Total ERK1/2 was subsequently probed and served as loading control. Semi-quantitative analysis of band density was calculated for statistic analyses using one-way ANOVA. Different alphabet letters show significant differences ($P < 0.05$) of phospho-protein/total protein ratio in time course. The *Error bars* represent mean ± SE.

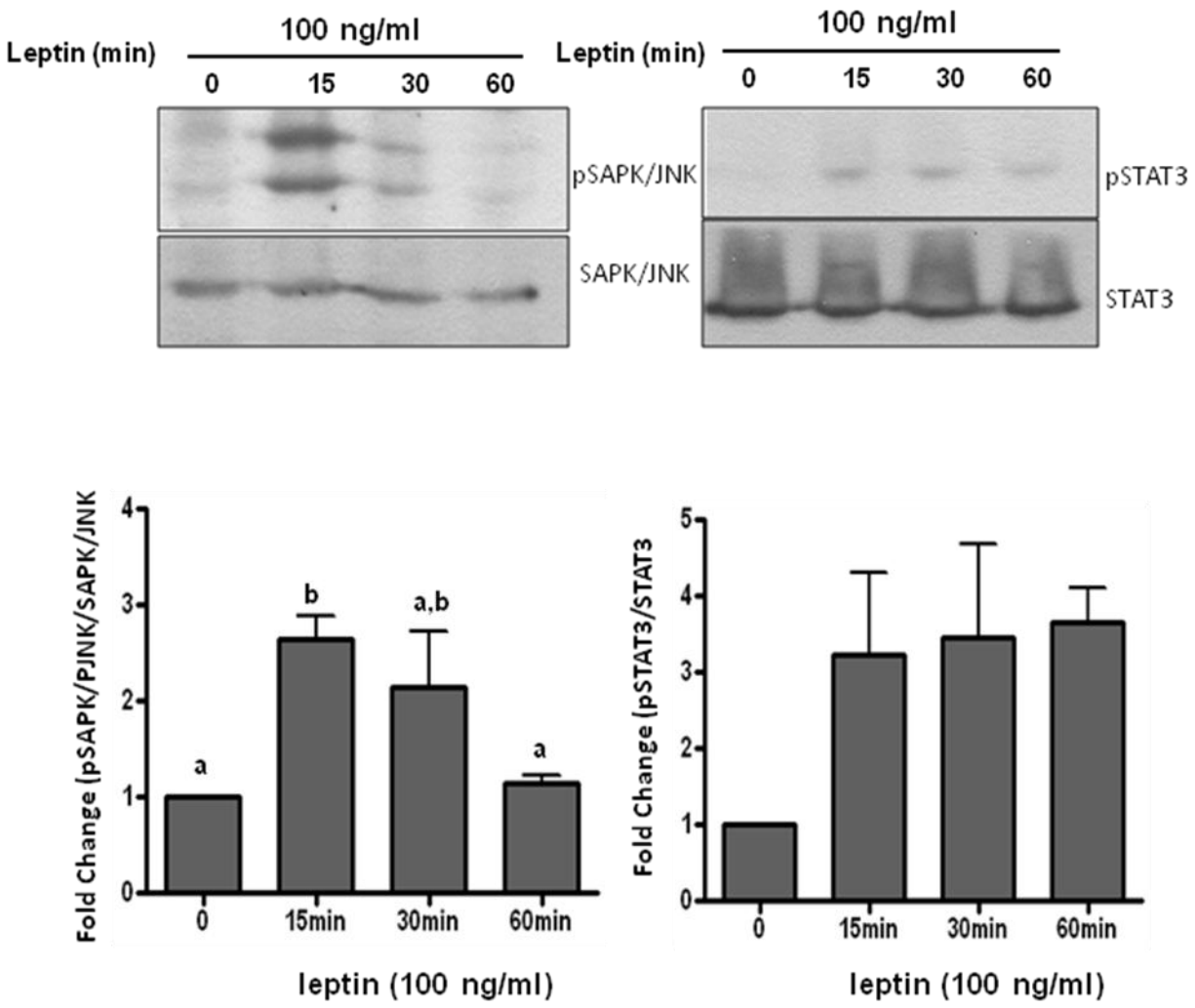

Figure 3.4. **Leptin activation of SAPK/JNK phosphorylation and STAT3 phosphorylation.** 500,000 HeLa cells were cultured in DMEM maintenance media in 10 cm dish. After 24 h serum starvation, cells were treated with 100ng/ml Leptin and then protein extracts were collected from HeLa cells treated with Leptin for 0, 15, 30 or 60 minutes. Western blot analysis was carried out using antibodies against phosphorylated pSAPK/JNK or phosphorylated pSTAT3. Total SAPK/JNK or total STAT3 was subsequently probed and served as loading control. Semi-quantitative analysis of band density was calculated for statistic analyses using one-way ANOVA. Different alphabet letters show significant differences ($P < 0.05$) of phospho-protein/total protein ratio in time course. The *Error bars* represent mean ± SE.

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CHAPTER 4

Regulation of Immediate Early and EMT Markers Genes by Pro-inflammatory cytokines in HeLa Cells

ABSTRACT

Obesity is associated with a chronic inflammatory response characterized by over-expression of cytokines including TNFα and IL-6. Thus, there may be an important relationship between abnormal chronic cytokine levels and cancer risk. To determine the role of TNFα and IL-6 on the expression of genes associated with increased cancer risk in obesity, HeLa cells were treated with IL-6 or TNFα for 0-8 hours. Quantitative, real-time RT-PCR was carried out using primers against immediate early (IE) genes JUN, FOS, IL-8, IL-6, MCP-1, and CASP10; and epithelial-mesenchymal transition (EMT) marker genes SNAI1, SNAI2, Twist, JAG1, and ZEB2. IL-6 stimulated a significant transient increase in FOS, IL-8 and MCP-1. Interestingly, IL-6 also increased the gene expression of IL-6 in HeLa cells. Furthermore, SNAI1 mRNA abundance was significantly increased by IL-6. Likewise TNFα treatment caused a transient increase in JUN, FOS, IL-6 and IL-8. To assess more information of the activity of IL-6 and TNFα, Western blot analyses were carried out to study phosphorylation of ERK1/2, STAT3, and SAPK/JNK. Both IL-6 and TNFα stimulated phosphorylation of SAPK/JNK ERK1/2 and STAT3. In all, this study has identified a novel mechanism for important relationship between IL-6, TNFα and cancer risk in obesity. It would be another key to provide new method to care and prevent cancer.

INTRODUCTION

Too much adipose tissue in obesity is not only characterized by increased hormone level, but also with macrophage infiltration which are an important source of inflammation in this tissue. In fact, obesity is associated with a chronic inflammatory response characterized by over expressed cytokines such as IL-6 ,TNFα, inhibitor of NF-κB kinase b (IKKb), macrophage migration inhibitory factor, nerve growth factor, vascular endothelial growth factor, plasminogen activator inhibitor 1 and haptoglobin; increased acute-phase reactants, and activation of inflammatory signaling pathways (1, 2)

Interleukin 6 (IL-6) and tumor necrosis factor alpha (TNFα) are multifunctional cytokines involved in the regulation of the immune response, hematopoiesis, and inflammation. IL-6 is produced by numerous types of immune or immune accessory cells, such as monocytes, fibroblasts, lymphocytes, and glial cells. It is also produced by nonimmune cells such as adipose cells (5). Tumor necrosis factor alpha (TNFα) is a 26-kDa transmembrane protein that is cleaved into a 17-kDa biologically active protein that exerts its effects via type I and type II TNFα receptors. TNFα is a member of a group of cytokines involved in systemic inflammation and is a multifunctional cytokine playing a role in apoptosis, cell survival, inflammation and immunity. TNFα plays an important role in cancer, such as “cachectin” and T-lymphocyte differentiation factor (3, 4). In fact, TNFα also is able to inhibit tumorigenesis and viral replication.

At the beginning, IL-6 and TNFα were thought to be products of the immune system alone that had immune functions only. Yet, it has become increasingly apparent that the cytokines involving in both the nervous and endocrine systems. IL-6 and,TNFα have been shown to have endocrine as well as autocrine and paracrine roles. IL-6 is a

pleiotropic cytokine with many pathophysiologic roles in humans (6), which has drawn much attention in the endocrine field including those studying the endocrine properties of adipose tissue. Adipose tissue is recognized as endocrine organ to express and secrete a variety of bioactive peptides, known as adipokines including IL-6, TNFα, adiponectin, and leptin, which act at both the local (autocrine/paracrine) and systemic (endocrine) level (1, 2, 7). In adipose tissue, TNFα is produced by adipocytes and stromovascular cells (8). In fact, obesity is characterized chronic mild inflammation, with increased circulating levels of inflammatory cytokine and the expression and release of inflammation-related adipokines (1). Recently, it has been shown that IL-6 and TNFα are both produced by the adipose tissue (5, 9), suggesting that they may play a key roles in lipid metabolism.

Cytokines are known to have both tumor-promoting and inhibitory effects on breast cancer growth depending presumably on their relative concentrations and the presence of other modulating factors (10). These bioactive peptides produced the adipose cell promote angiogenesis including vascular endothelial growth factor, hepatocyte growth factor, leptin, tumour necrosis factor-alpha, heparin-binding epidermal growth factor-like growth factor, and interleukin-6 (11). It is well known that there is an increased cancer risk by obesity and there is growing evidence that tumors are sustained and promoted by inflammatory signals from the surrounding microenvironment. Together with the unbalance of hormones and factors which are produced by the adipose cells in obesity, there may have a link between the adipose cells produced pro-inflammatory cytokine and the increased risk of cancer. IL-6 and TNFα may play the key role in increased cancer risk in obesity.

Interleukin-6 (IL-6) is a pleiotropic cytokine with obviously tumor-promoting and tumor-inhibitory effects (10). Knüpfer has reported that, IL-6 would be a negative prognosticator in breast tumor patients concerning patients' serum IL-6 levels (10). Grivennikov and Bollrath demonstrated that the importance of the interleukin-6 family of proinflammatory cytokines and their downstream effector STAT3 in colitis-associated colon cancer (12, 13). Moreover, TNFα acts as a multifunctional cytokine to involve in apoptosis, cell survival, inflammation, and immunity (14, 15). Recently, TNFα is used in the regional treatment of locally advanced soft tissue sarcomas and metastatic melanomas and other tumors (16).

In all, the pro-inflammatory cytokine IL-6 andTNFα would be a new key to understand the link between obesity and cancer. We need to drew more attention to the relationship between pro-inflammatory cytokine and cancer, such as IL-6 andTNFα in obesity

MATERIALS AND METHODS

HeLa cell culture and whole cell protein extracts: 500,000 HeLa cells (American Type Culture Collection, Manassas, VA) were cultured in 10 cm dish with Eagle's Minimum Essential Medium pH 7.4 (Sigma, St. Louis, MO), containing 1.5 g/L NaHCO3, 0.11 g/L Sodium pyruvate, 0.292 g/L L-glutamine, 10 ml/L Penn-strep, 1 ml/L phenol red and 100 ml/L of heat inactivated FBS (Hyclone, Logan, UT, USA). After 24h, the cells were changed to the same medium except there was no FBS included for serum starvation. After 24h serum starvation, cells were treated with 10 ng/ml interleukin-6 (Cell Signaling Technology, Danvers, MA) or 10 ng/ml TNFα (Cell Signaling

Technology, Danvers, MA) for 0, 15, 30 or 60 minutes. The cells were washed with 1X HBSS (Invitrogen, Carlsbad, CA, USA) and cells collected in RIPA buffer (150 mM NaCl, 1 mM EDTA, 50mM Tris-HCl pH7.4, 1% NP-40, 0.25% Na-deoxycholate) containing phosphatase inhibitors (1 mM NaF and 1 mM Na3VO4) and Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics). Cells in the RIPA buffer were sonicated 15 seconds and then centrifuged at 10,000 x g for 5 minute to separate cell debris and the supernatant which contain soluble protein. The protein concentration of each experimental replicate was determined with the Pierce BCA Protein Assay (Rockford, IL, USA).

HeLa cell culture and RNA extraction: 200,000 HeLa cells (American Type Culture Collection, Manassas, VA) were cultured in 3.5 cm dish and serum starved for 24 hours as described above. After serum starvation, cells were treated with 10 ng/ml IL-6 or 10 ng/ml TNFα (Cell Signaling Technology) for 0, 0.25, 0.5, 1, 2, 4, or 8 hours. The cells were washed with 1X HBSS (Invitrogen, Carlsbad, CA, USA) and then collected in TRI reagent (Ambion Inc., Austin, TX, USA). Total RNA was extracted according to the manufacturer's protocol (Sigma, St. Louis, MO). After isolation, the total RNA was dissolved in 20 µl diethyl pyrocarbonate (DEPC) water. The RNA concentration was determined using Beckman Coulter DU 730 Life Science UV/Vis Spectrophotometer.

Reverse Transcription: Total HeLa cell RNA (5 µg) was mixed with 5 units of RQ1 RNase free DNase (Promega, Madison, WI), M-MLV RT buffer and DEPC water and incubated at 37°C for 30 minutes to remove genomic DNA contaminants. Before RT-PCR, 1.1 µl of RNA and RQ1 mix was removed from each sample to a new tube with 15 µl DEPC water, which should be used as No RT to detect the residual genomic DNA.

The mixed RNA samples was subsequently combined with 2 µl Random primers (Promega), 2 µl dexoxynucleotide triphosphates (Fermantas; 10 mM dNTP mix), 2.4 µl DEPC water, and then were incubated in 65°C for 5 min. After that, the mixed samples were chilled on the ice quickly. 400 units of Moloney Murine Leukemia Virus reverse transcriptase (Promega), 3.2 µl of RT buffer (Invitrogen, Carlsbad, CA), and 2 µl 0.1 M DL-dithiothreitol (DTT) (Invitrogen, Carlsbad, CA) were added into each sample. The mixed samples were incubated at 37°C for 2 hour and followed by 15 min at 72°C to stop the reverse transcriptase. The cDNA was stored at -20°C for subsequent real time-PCR.

Quantitative, Real-Time PCR (qPCR) Analysis: Gene-specific forward and reverse primers were designed (Primer Express, Applied Biosystems, Foster City, CA) and synthesized (Integrated DNA Technologies, Coralville, IA) and store at -20°C. The information of the primers and probes are shown in table 4.1. Each set of gene-specific primers was tested to determine the maximal concentration of primers that could be used to produce specific amplification of the target sequence without primer dimer amplification. Before use, each cDNA sample was diluted 1:10. Glyceradehyde-3-phosphate dehydrogenase (Gapdh) gene is a housekeeping gene and used as a control for reverse transcription efficiency and well-to-well variation in template. PCR was performed with 10 µl Taqman Universal Master Mix (Applied Biosystem, Foster City, CA), 1 µl cDNA, 1 µl probe with primers, and 8 µl DEPC to make up the reaction volume to 20 µl. QRT-PCR was performed in 364 well plates (Axygen Scientific, Union City, CA) with an adhesive cover film (VWR, Scientific Products, North Kansas City, MO) in 7900HT Fast Real-Time PCR system (Applied Biosystems). Quantitative PCR (qPCR) reactions of the other target genes were carried out using 1:10 dilutions of each

cDNA sample and standard samples with Power SYBR Green PCR Master Mix (Applied Biosystem, Foster City, CA). All experimental and Gapdh PCRs were carried out in separate wells in triplicate. These relative values are plotted against the threshold value for each dilution to generate a standard curve. An arbitrary value of template was assigned to the highest standard and corresponding values were assigned to the subsequent dilutions. According to the slope and y-intercept of the standard curve, 7900HT Fast Real-Time PCR system assigned a value of the relative amount for each experimental and Gapdh triplicate. The value of the average of the experimental triplicate divided by the average of the Gapdh triplicate was used for statistical analysis.

Western Blot Analyses: The protein samples were resolved by SDS-polyacrylamide 10% gel electrophoresis which contains 4% stacking gel and 10% separating gel. Protein samples were loaded together with loading buffer and the separated protein was transferred to Immobilon PVDF (Millipore, Billerica, MA). Following transfer, the membranes were blocked with 5% nonfat dry milk in 1X TBST (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 01% (v/v) Tween 20) for 1 hour with gentle shaking in order to block the nonspecific proteins. After that, the blots were probed with the primary antibodies which are diluted in 5% w/v BSA, 1XTBS, 0.1% Tween 20. The membrane was then incubated with primary antibody against phospho-AKT (Cell Signaling Technology, Danvers, MA), phospho-ERK1/2 (Cell Signaling Technology), phospho-SAPK/JNK (Cell Signaling Technology), or phospho-STAT3 (Cell Signaling Technology) overnight at 4°C with gentle shaking. Blots were washed with 1X TBST and incubated for 1 hour with HRP-conjugated secondary antibody which was diluted with 5% nonfat dry milk in 1X TBST (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 01%

(v/v) Tween 20). Proteins were incubated with West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) for 5 min and exposed to autoradiograph film (Fisher Scientific, Fairlawn, IL) in dark room. After the visualization of phosphorylated protein, the blots were stripped with Restore plus Western Blot Stripping Buffer (Pierce) in room temperature for 20 min and 37°C 10 min. Wash the blots with 1X TBST and blocked with 5% milk in 1XTBST. Next, incubated with primary antibody against total AKT, total ERK1/2, total SAPK/JNK, or total STAT3 (Cell Signaling Technology) overnight at 4°C with gentle shaking. Total protein was exposed and visualized as described above. The visualized total protein served as a loading control for each sample. The autoradiograph images films were scanned and the density of the protein band was determined in Photoshop. Semi-quantitative analysis of band density of the phosphorylated and total protein was calculated in each film as described by Miller (http://lukemiller.org/journal/2007/08/quantifying-western-blots-without.html). The amount of phosphorylated protein was normalized by total protein expressed in each sample. The normalized abundance of phosphorylated protein of each sample was subsequently compared to the untreated control sample. The data was showed as a fold-change.

Statistical Analyses: All statistical analyses were carried out by GraphPad Prism 4.0 (Graphpad Software, La Jolla, CA). QPCR data and western-blot data were analyzed using one-way ANOVA. Differences in data were considered statistically significant at $P < 0.05$. Different letters indicate significant differences in fold change in mRNA abundance compared to control for all qPCR data and phospho-protein/total protein ratio in time course. The Error bars represent mean ± SE.

Results

Significant gene expression differences In HeLa cells by IL-6

To determine the effects of IL-6 on the regulation of immediate early genes, HeLa cells were treated with 10ng/ml of IL-6 in a time course after 24h serum starvation. FOS, IL-8 and MCP-1 have significant elevated mRNA expression level early in 1 hour, as compared to untreated control cells. FOS mRNA increased by 2 fold, IL-8 by 7 fold, and MCP-1 by 2 fold, respectively. After these peak increases in mRNA abundance, their mRNA expression decreased quickly. Interestingly, IL-6 is able to induce its own mRNA with an increase of 2 fold early at 0.5 hour. IL-6 mRNA induction arrived at peak at 1 hour, and dropped back to control level at 2 hours. However, IL-6 didn't lead to significant induction on JUN and caspase 10 (Fig 4.1). The role of IL-6 on mRNA induction of epithelial-mesenchymal transition marker genes was also assessed in the IL-6 treated HeLa cells. SNAI1 mRNA was induced to 1.6 times control level. However, IL-6 had no significant effect on mRNA level of SNAI2, ZEB2, TWIST1 and JAG1 (Fig 4.2).

Signaling Pathways regulated by IL-6 in HeLa cells

Here, we tested if 10ng/ml of IL-6 changes phosphorylation of ERK1/2 and JNK-STAT. Fig 4.3 showed that 10ng/ml IL-6 activated ERK1/2 by increasing phosphorylation of ERK1/2 dependent of time. Early in 15 minutes, phosphorylation of ERK1/2 increased by 4 fold in the induction of IL-6. In Fig 4.4, 10ng/ml IL-6 activated SAPK/JNK by increasing their phosphorylation by 2.5 fold in 15 minutes. But phosphorylation of SAPK/JNK decreased quickly by 30 minutes post-treatment. In

contrast, 10ng/ml IL-6 activated STAT3 by increasing its phosphorylation by 6 fold in 15 minutes and p-STAT-3 began to decrease after 30 minutes.

Significant gene expression differences In HeLa cells by TNFα

We also determined the effects of another important inflammation factor, TNFα, on the regulation of immediate early genes. HeLa cells were treated with 10ng/ml of TNFα in a time course after 24h serum starvation (Fig 4.5). JUN, FOS and IL-6 had significant elevated mRNA expression level 30 minutes after treatment was initiated compared to untreated control cells. JUN mRNA increased by 4-fold, FOS by 7-fold, and IL-6 by 2-fold. TNFα (10ng/ml) was also able to increase IL-8, MCP-1 mRNA at 1 hour. However, 10ng/ml of TNFα didn't lead to significant induction on pro-apoptotic factor caspase 10. Fig 4.6 indicated that TNFα was also not able to lead to significant induction of SNAI1, SNAI2, ZEB2, or TWIST1 mRNA, whereas JAG1 mRNA expression was increased 2 hours post-treatment.

Signaling Pathways regulated byTNFα in HeLa cells

Here, we tested if 10ng/ml of TNFα changes phosphorylation of ERK1/2 and JNK-STAT. Fig 4.7 showed that 10ng/mlTNFα activated ERK1/2 by increasing phosphorylation of ERK dependent of time. Early in 15 minutes, phosphorylation of ERK1/2 increased by 5.5 fold in the induction of TNFα. However, phosphorylation of ERK1/2 got weaker and weaker after that. In Fig 4.7 and Fig 4.8, 10ng/ml TNFα activated SAPK/JNK by increasing their phosphorylation by 7 fold in 15 minutes. But phosphorylation of SAPK/JNK decreased quickly in another 15 minutes. In contrast, 10ng/mlTNFα activated STAT3 by increasing its phosphorylation by 1 fold in 15

minutes. Interestingly, p-STAT-3 kept increasing. At 1 hour, p-STAT-3 increased by 3.5 fold.

Discussion

Inflammation is recognized to play important role in the pathogenesis of many types of malignancies. Now, more and more attention has been focused on the role cytokine of NF-κB, IL-6, TNFα and their signal pathway in mediating the link between inflammation and cancer. Michael Karin's group reported the elevated levels of IL-6 in murine models of colitis-associatedcancer (CAC) (17). The higher serum levels of IL-6 exhibited in males could be the reason of gender bias in liver cancer susceptibility (18). In our data, with the treatment of IL-6 in HeLa cell, there is a increased IL-6 mRNA level. It seems as a positive feedback of IL-6 in inflammation. However, IL-6 promotes the in vivo growth of tumors in models of prostate, breast, and lung cancers (10). The increased IL-6 may be the important link between inflammation and cancer, which promotes the tumorigenesis.

Our data also demonstrated that IL-6 is able to increase FOS mRNA level in 0.5 hour in HeLa cells. However, the JUN mRNA level was not induced significantly. Therefore in our experiments, it is hard to evaluate the induction of AP-1 through IL-6 treatment. Apparently, as a pro-inflammatory factor, IL-6 is able to induce other cytokines' transcription, including IL-8, MCP-1 in our data. IL-8 has been shown to contribute to human cancer progression through its potential functions as a mitogenic, angiogenic, and motogenic factor. IL-8 expression is regulated by various tumor microenvironment factors, such as hypoxia, acidosis, nitric oxide, and cell density (19). Le reported that IL-8 plays an important role in tumor angiogenesis and contributes

significantly to the aggressive biology of human pancreatic cancer (20). Moreover, point-mutation of NF-κB, AP-1, or IL-6 binding sites significantly reduced or eliminated the constitutive IL-8 promoter activity (20). As induced expression by IL-6 in HeLa cell, the increased IL-8 may stimulate tumorigenesis. MCP-1 is a relevant negative regulator of pancreatic cancer progression (21). All primary tumors were tested, and 6 of 14 pancreatic cancer cell lines were constitutively secreted MCP-1. The increased MCP-1 in HeLa cell may be produced by the high activated HeLa cell or induced by pro-inflammatory cytokine which produced by HeLa cell or the treatment of IL-6.

IL-6 is an important cytokine, which acts as both a pro-inflammatory and anti-inflammatory factor. IL-6-type cytokines bind to plasma membrane receptor complexes, then induce transducing receptor chain gp 130 (glycoprotein 130), then activate the associated Janus kinases (JAKs). STAT3 is a nuclear transcription factor downstream of gp130. JAKs phosphorylate gp130, leading to the recruitment and activation of the STAT3 and STAT1 transcription factors as well as other molecules (SHP2, Ras-MAPK, and PI3K) (22). As Our western-blot data in HeLa cell, IL-6 activate phosphorylation of ERK1/2, SAPK/JNK, and STAT3 early at 15 minutes, with continued increased phosphorylation of STAT3 at 30min. As reported by Grivennikov and Bollrath, STAT is necessary for the growth of colitis-associated colorectal cancer in mice (12, 23). STAT3 could induce and maintain a procarcinogenic inflammatory microenvironment, both at the initiation of malignant transformation and during progression of cancer (12, 24, 25). STAT3 is linked to inflammation associated tumorigenesis through genetic alterations in malignant cells (26-28). In fact, STAT3 is frequently activated in malignant cells and capable of inducing a large number of genes which are important for inflammation (29).

In fact, STAT3 signalling is a major intrinsic pathway for cancer inflammation. IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. Take together, ERKs, JNK, STAT3 may be widely expressed protein kinase intracellular signalling molecules that are involved in meiosis, mitosis.

It is reported that an inflammatory stimulus induces production of TNFα, and then TNFα could primarily induce production of Il-1, IL-6, IL-8 and other pro-inflammatory mediators (30, 31). In our experiment, we also used TNF alpha as the inducer in HeLa cells. TNF alpha is able to lead to much stronger induction than did IL-6. In other words, TNF alpha is sufficient to activate downstream genes in mRNA level. With TNFα treatment, IL-6 had significant elevated mRNA expression level early in 0.5 hour, as compared to control, while IL-8, MCP-1 mRNA with increase of 2 fold, 0.6 fold respectively at 1 hour. The treatment of TNFα induces the production of IL-6, IL-8 and MCP-1. The increased IL-6 and IL-8 may promote the tumorigensis as discuss above.

In addition, TNFα induce the increase of JUN mRNA by 4 fold, FOS by 7 fold in HeLa cell. It is established that AP-1 proteins participate in tumorigenesis by regulating oncogenic transformation, proliferation, apoptosis, invasive growth and angiogenesis. Fos and FosB are dispensable for cell cycle progression, for example, fibroblasts and embryonic stem cells have proliferation defect if lacking these components (32-34). Moreover, over-expression of c-Fos induces cell death and resistant to apoptosis in an embryonic Syrian hamster cell line (35). Over-expressed c-Fos transforms in chondroblasts and osteoblasts, can induced the c-Fos-induced tumorigenesis (36, 37). In fact, dependent on its ability to heterodimerize with Jun proteins and to bind to DNA, c-

Fos acts as oncogenic. The dramatic induction of JUN and FOS mRNA level caused by TNFα in HeLa cells may cause promotion of tumor.

However, TNFα is commonly used to treat cancer in clinical for it can be pro-angiogenic and used clinically to destroy tumor vasculature (38). Recently,TNFα is used in cancer in the regional treatment of locally advanced soft tissue sarcomas and metastatic melanomas and other tumors (16). TNFα is a potent anti-vascular cytokine at higher doses. But TNF-α has powerful and toxic systemic side effects at the high dose which limited uses in clinical (39). Paradoxically, TNFα is highly expresses in tumor and recognized as pro-angiogenic with low dose (39). Although over the last few decades high-dose administration of TNF-a has been used as a cytotoxic agent, recent pre-clinical cancer models have support that chronic, low level TNF-a exposure cause the acquisition of pro-malignant phenotype including increased growth, invasion and metastasis (40). The growth of new capillaries into the tumor is called 'tumorangiogenesis' (41, 42). Angiogenesis is induced by the release of various proangiogenic cytokines by the tumor cells and the supporting cells (43). Pro-angiogenic factors involve in endothelial cell proliferation and migration, the formation of endothelial cells into new vasculature, and the degradation of the basement membrane and the extracellular matrix (43), which are important for the tumor growth. Low dose of TNFα works as pro-angiogenic factor and may promote the tumorigenesis. The 10ng/ml TNFα in HeLa cell induce so many pro-inflammatory factor which may promote the tumorigenesis and the low dose may also work as a pro-angiogenic factor and tumor-promoter to have positive function to tumor growth.

In the signal pathway,TNFα activated ERK1/2 by increasing phosphorylation of ERK1/2 early at 15 minutes by 5.5 fold in the induction ofTNFα. While the phosphorylation SAPK/JNK increase by 7 fold at 15 minutes. However, 10ng/mlTNFα activated STAT3 by increasing its phosphorylation from 15 minutes to 1 hour. This provides a clue that ERK and JNK-STAT are mediators to deliverTNFα signal to increase some downstream target gene expression. Stat3 can be a link between inflammation and cancer. STAT3 could induce and maintain a procarcinogenic inflammatory microenvironment, both at the initiation of malignant transformation and during progression of cancer (12, 24, 25). STAT3 is linked to inflammation associated tumorigenesis through genetic alterations in malignant cells (26-28). In cancer cell, Grivennikov and Bollrath demonstrate that STAT3 is necessary for the growth of colitis-associated colorectal cancer in mice (12, 23). It may provide another key to understandTNFα as a pro-inflammation cytokine and its function in tumorigenesis.

In normal conditions, cytokines are known to have both tumor-promoting and inhibitory effects on breast cancer growth depending presumably on their relative concentrations with presence of other modulating factors (10). In fact, obesity is associated with a chronic inflammatory response characterized by over expressed cytokine such as IL-6 ,TNFα, adiponectin, inhibitor of NF-kB kinase b (IKKb), macrophage migration inhibitory factor, nerve growth factor, vascular endothelial growth factor, plasminogen activator inhibitor 1 and haptoglobin; increased acute-phase reactants, and activation of inflammatory signaling pathways (1, 2). There is an increased expression ofTNFα in adipose tissue in human obesity (44). The adipose cell produced bioactive peptides including vascular endothelial growth factor, hepatocyte growth

factor, leptin, tumor necrosis factor-alpha, heparin-binding epidermal growth factor-like growth factor, and interleukin-6 (11). The inflammatory conditions in obesity may initiate or promote oncogenic transformation, and also can generate an inflammatory microenvironment that promotes tumour progression. Take together, the effects and function of the elevations of the cytokines in obesity may be a link to understand more about the obesity related cancer. To understand the important roles of TNF-a and IL-6 in the process of tumor promotion will assist in the development of cancer therapeutics.

Table 4.1. Human Primer sequences used for QPCR analysis

Gene	**Probe Dye**	**Primer**	**Sequence (**5' to 3'**)**
CASP10	SYBR Green	Forward	GGAGCTGTCTACTCTTCGGATGA
		Reverse	AGGGCTGTGAAGTGAGACATGAT
FOS	SYBR Green	Forward	CCTCGCCCGGCTTTG
		Reverse	GCCTCGTAGTCTGCGTTGAAG
JUN	SYBR Green	Forward	CTGGGAGGACCGGAGACA
		Reverse	GAGAAGCCTAAGACGCAGGAAA
MCP-1	SYBR Green	Forward	CAAGCAGAAGTGGGTTCAGGAT
		Reverse	TCTTCGGAGTTTGGGTTTGC
IL-6	SYBR Green	Forward	AGGGCTCTTCGGCAAATGTA
		Reverse	GAAGGAATGCCCATTAACAACAA
IL-8	SYBR Green	Forward	CTGGCCGTGGCTCTCTTG
		Reverse	CCTTGGCAAAACTGCACCTT
SNAI1	SYBR Green	Forward	CCCCAATCGGAAGCCTAACT
		Reverse	GCTGGAAGGTAAACTCTGGATTAGA
SNAI2	SYBR Green	Forward	CCTGGGCGCCCTGAA
		Reverse	TTCTCCCCCGTGTGAGTTCT
TWIST1	SYBR Green	Forward	TCCGCGTCCCACTAGCA
		Reverse	AGTTATCCAGCTCCAGAGTCTCTAGAC
ZEB2	SYBR Green	Forward	CCAGCTCGAGCGGCATA
		Reverse	GCCACACTCTGTGCATTTGAA
JAG1	SYBR Green	Forward	CAGCTCTGTGACAAAGATCTCAATTAC
		Reverse	AGGGCCTGTGTTGCTACAAGTT
GAPDH	Kit-Taqman Rodent GapDH Control Reagents Applied Biosystems (VIC Probe)		

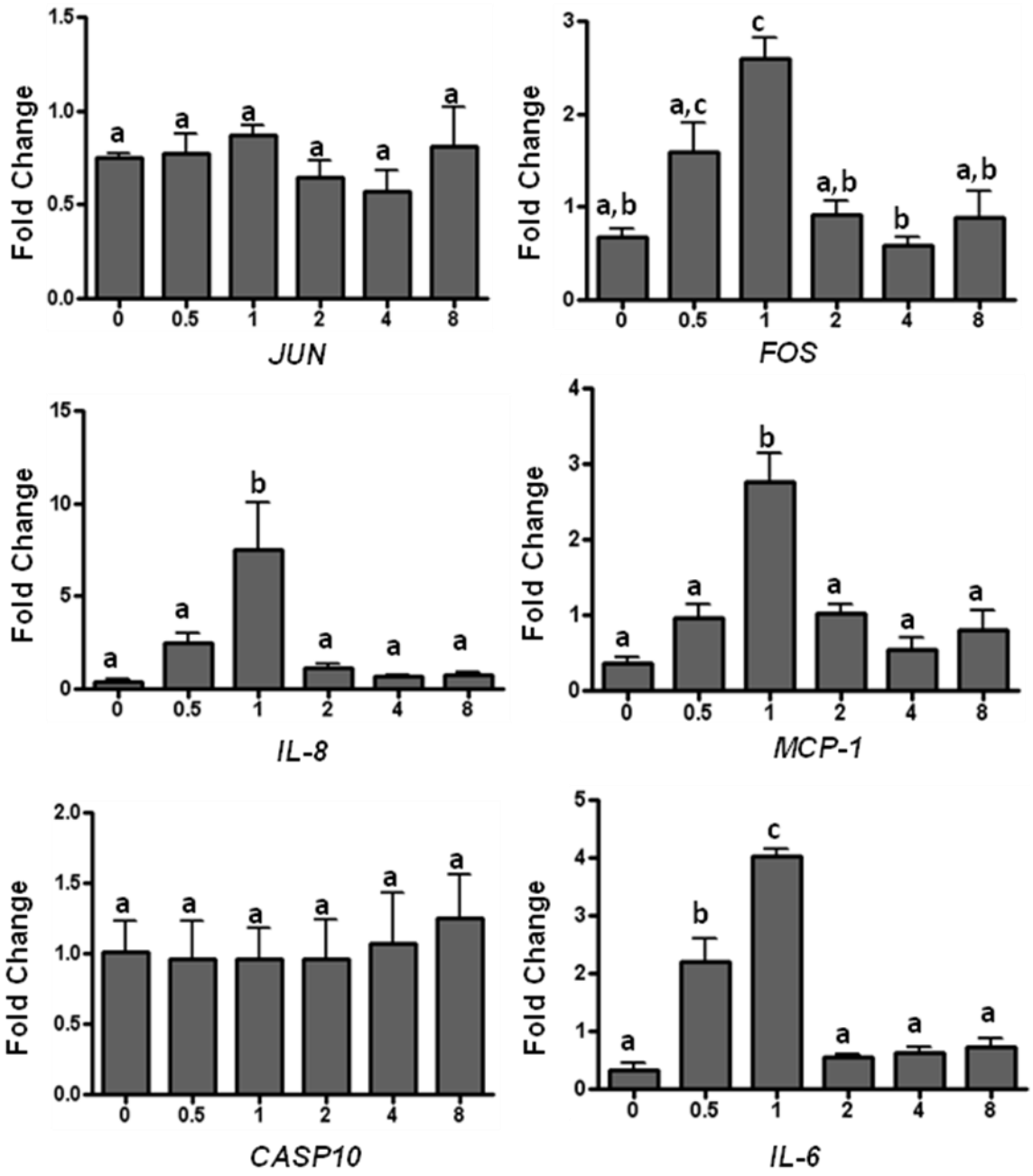

Figure 4.1. **Il-6 dependent regulation of immediate early genes expression of HeLa cells.** 200,000 HeLa cells were cultured in DMEM maintenance media in 3.5 cm dish. After 24 h serum starvation, cells were treated with 10 ng/ml IL-6 for 0, 0.5, 1, 2, 4, or 8 h. Cells were collected. Isolated the RNA and QPCR was carried out using primers against *JUN, FOS, IL-8, IL-6* and *CASP10*. The mRNA abundance of each candidate in each sample was normalized by GAPDH mRNA abundance and expressed as a fold change. Comparing the candidate gene expression in different time points. All QPCR data was tested for significant differences in mRNA abundance using one-way ANOVA. Alphabet letters show significant fold change ($P < 0.05$). *Error bars* represent mean ± SE.

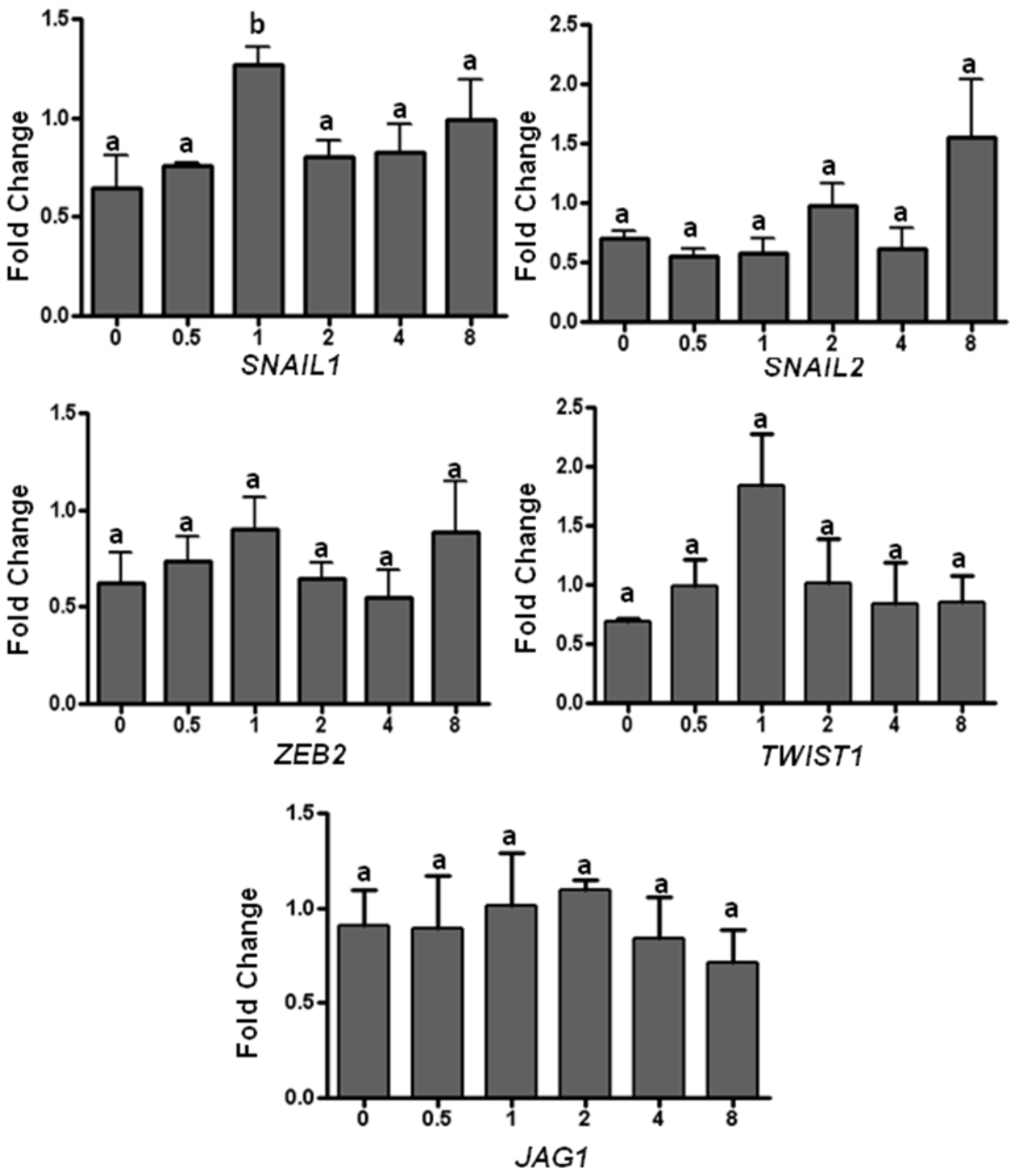

Figure 4.2. **Il-6 dependent regulation of epithelial transition markers genes expression of HeLa cells.** 200,000 HeLa cells were cultured in DMEM maintenance media in 3.5 cm dish. After 24 h serum starvation, cells were treated with 10ng/ml IL-6 for 0, 0.5, 1, 2, 4, or 8 h. Cells were collected. Isolated the RNA and QPCR was carried out using primers against *SNAI1, SNAI2, TWIST, CDH1, ZEB2,* and *JAG1*. The mRNA abundance of each candidate in each sample was normalized by GAPDH mRNA abundance and expressed as a fold change. Comparing the candidate gene expression in different time points. All QPCR data was tested for significant differences in mRNA abundance using one-way ANOVA. Alphabet letters show significant fold change ($P < 0.05$). *Error bars* represent mean ± SE.

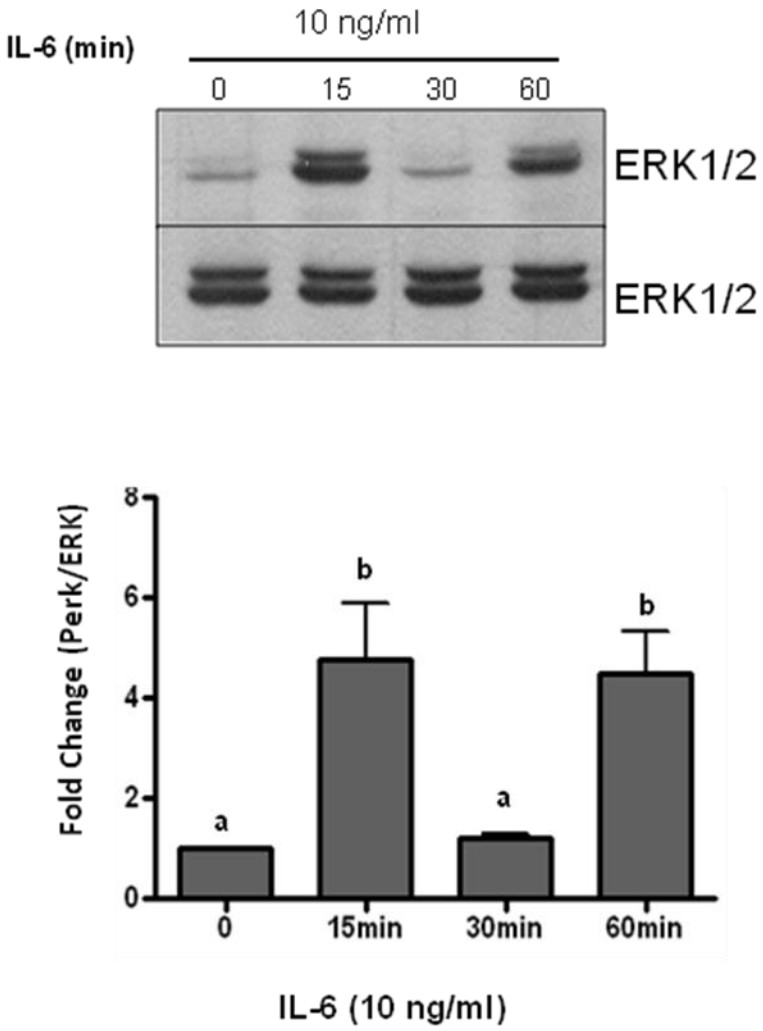

Figure 4.3. **IL-6 activation of ERK1/2 phosphorylation.** HeLa cells were cultured with DMEM maintenance media in 10 cm dish. After 24 h serum starvation, cells were treated with 10 ng/ml IL-6 and then protein extracts were collected from HeLa cells treated with IL-6 for 0, 15, 30 or 60 minutes. Western blot analysis was carried out using antibodies against phosphorylated pERK1/2. Total ERK1/2 was subsequently probed and served as loading controls. Semi-quantitative analysis of band density was calculated for statistic analyses using one-way ANOVA. Different alphabet letters show significant differences ($P < 0.05$) of phospho-protein/total protein ratio in time course. The *Error bars* represent mean ± SE.

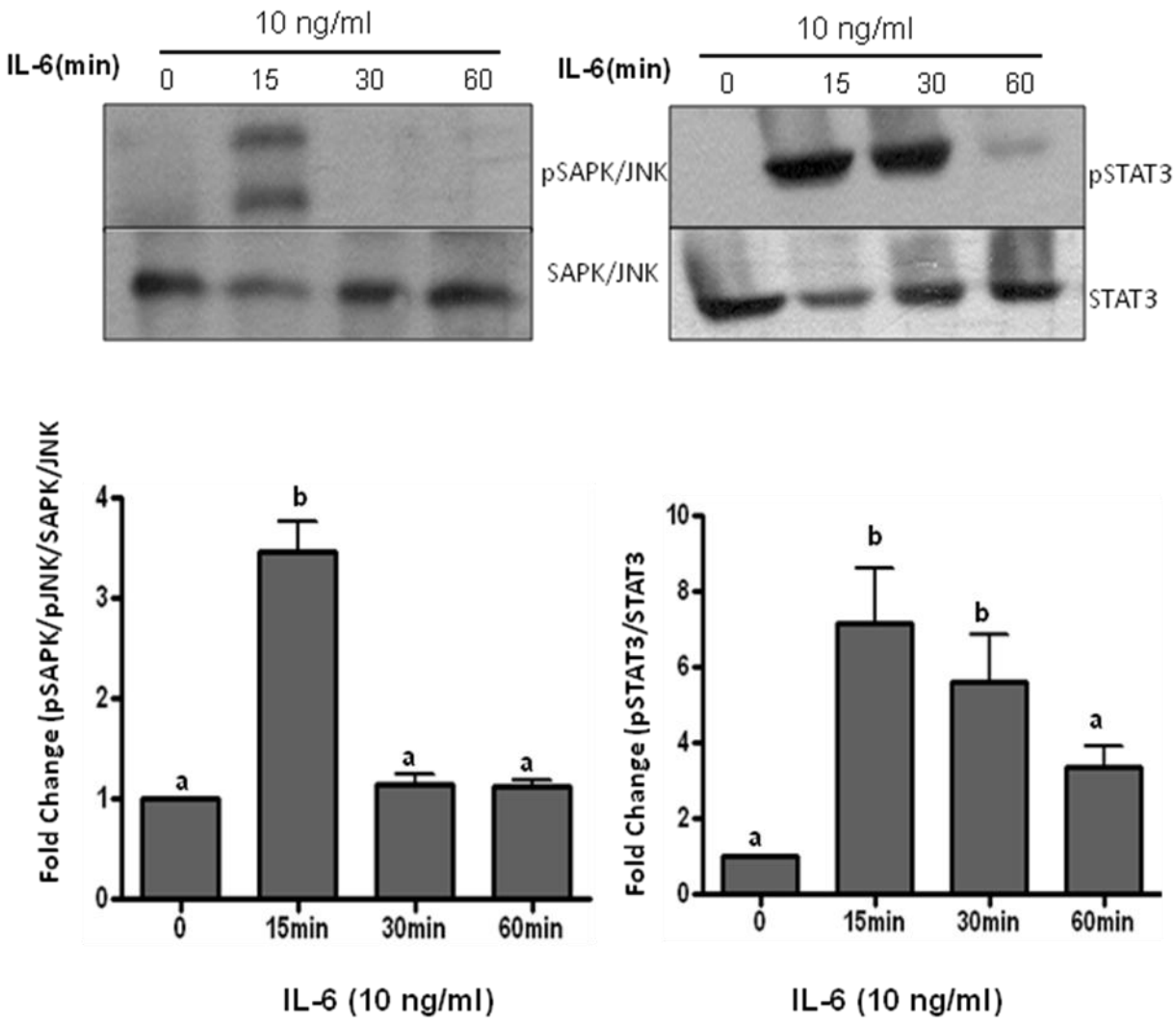

Figure 4.4. **IL-6 activation of SAPK/JNK phosphorylation and STAT3 phosphorylation**. 500,000 HeLa cells were cultured with DMEM maintenance media in 10 cm dish. After 24 h serum starvation, cells were treated with 10 ng/ml IL-6 and then protein extracts were collected from HeLa cells treated with IL-6 for 0, 15, 30 or 60 minutes. Western blot analysis was carried out using antibodies against phosphorylated pSAPK/JNK or phosphorylated pSTAT3. Total SAPK/JNK or total STAT3 was subsequently probed and served as loading controls. Semi-quantitative analysis of band density was calculated for statistic analyses using one-way ANOVA. Different alphabet letters show significant differences ($P < 0.05$) of phospho-protein/total protein ratio in time course. The Error bars represent mean ± SE.

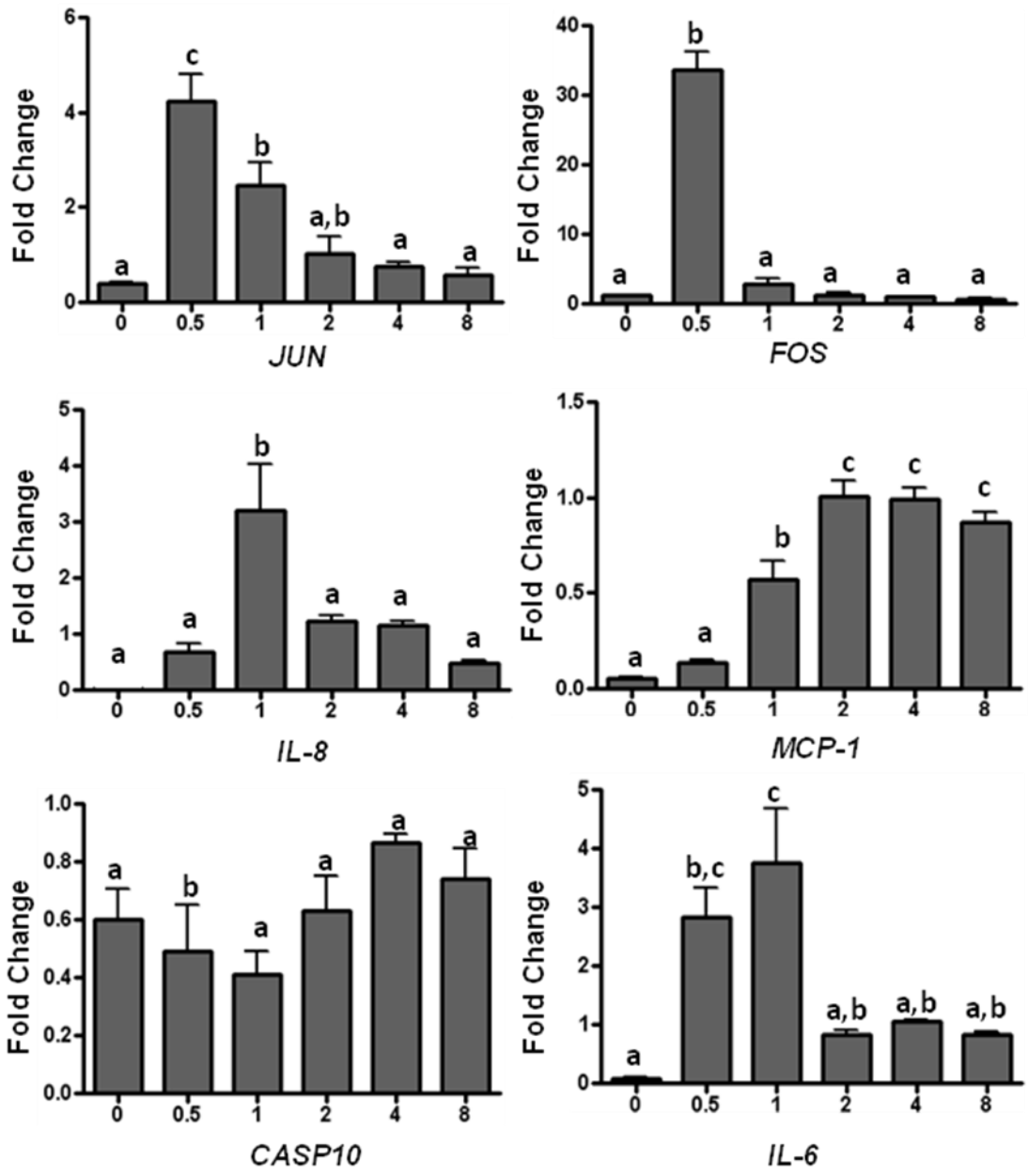

Figure 4.5. **TNFα dependent regulation of immediate early genes expression of HeLa cells.** 200,000 HeLa cells were cultured with DMEM maintenance media in 3.5 cm dish. After 24 h serum starvation, cells were treated with 10 ng/ml TNFα for 0, 0.25, 0.5, 1, 2, 4 or 8 h. Cells were collected. Isolated the RNA and QPCR was carried out using primers against *JUN, FOS, IL-8, IL-6* and *CASP10*. The mRNA abundance of each candidate in each sample was normalized by GAPDH mRNA abundance and expressed as a fold change. Comparing the candidate gene expression in different time points. All QPCR data was tested for significant differences in mRNA abundance using one-way ANOVA. Alphabet letters show significant fold change ($P < 0.05$). *Error bars* represent mean ± SE.

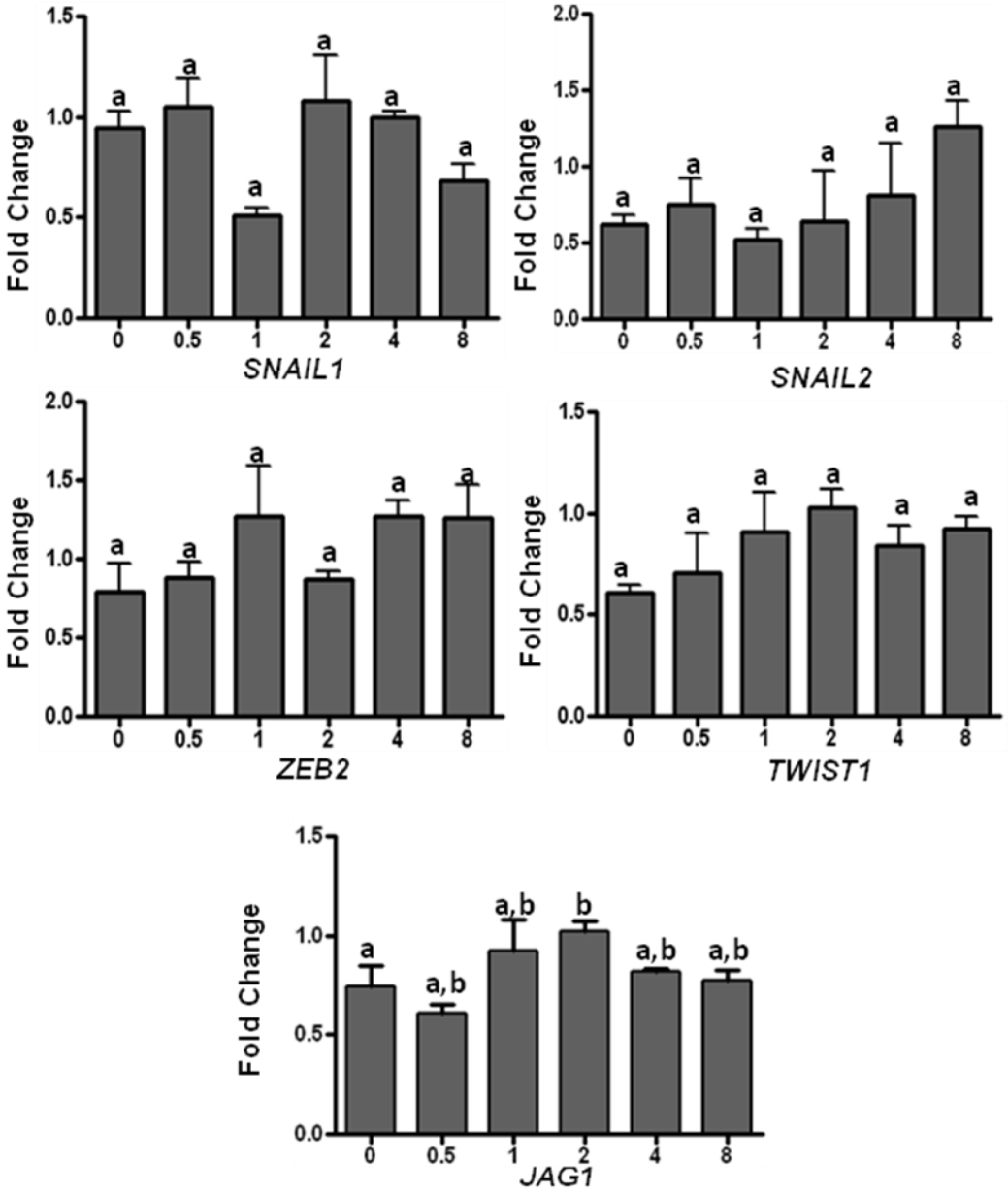

Figure 4.6. **TNFα dependent regulation of epithelial transition markers genes expression of HeLa cells.** 200,000 HeLa cells were cultured with DMEM maintenance media in 3.5 cm dish. After 24 h serum starvation, cells were treated with 10 ng/ml TNFα for 0, 0.25, 0.5, 1, 2, 4 or 8 h. Cells were collected. Isolated the RNA and QPCR was carried out using primers against *SNAI1, SNAI2, TWIST1, CDH1, ZEB2,* and *JAG1*. The mRNA abundance of each candidate in each sample was normalized by GAPDH mRNA abundance and expressed as a fold change. Comparing the candidate gene expression in different time points. All QPCR data was tested for significant differences in mRNA abundance using one-way ANOVA. Alphabet letters show significant fold change ($P < 0.05$). *Error bars* represent mean ± SE.

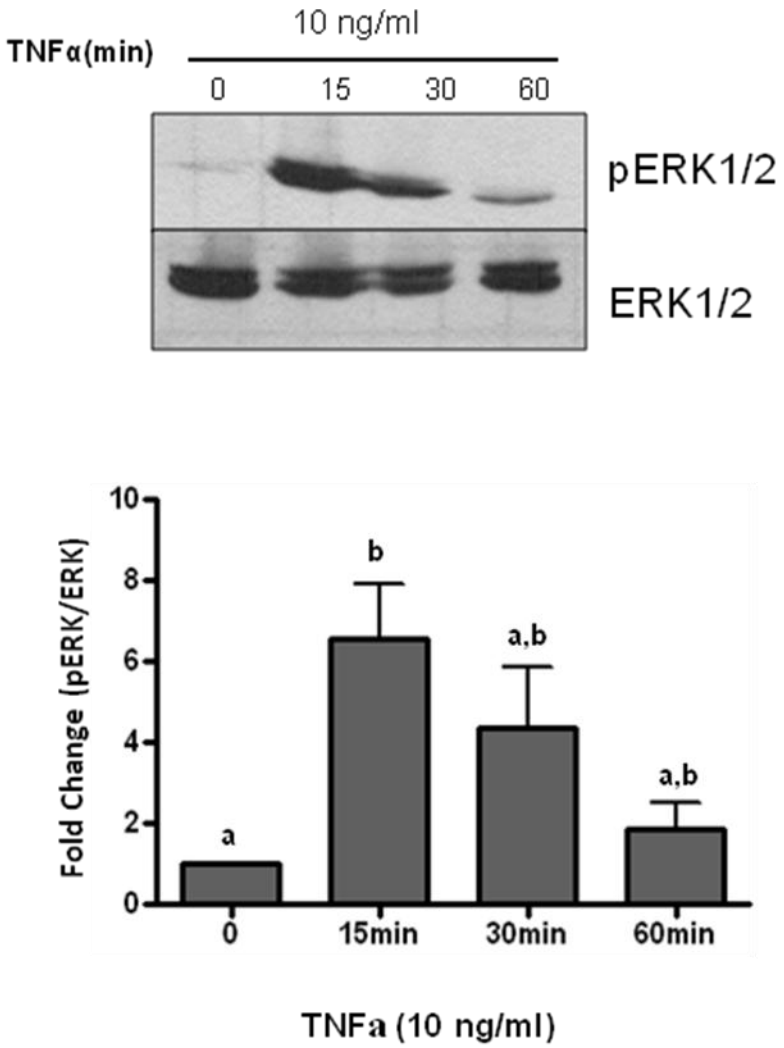

Figure 4.7. **TNFα activation of ERK1/2 phosphorylation**. HeLa cells were cultured in DMEM maintenance media in 10 cm dish. After 24 h serum starvation, cells were treated with 10 ng/ml TNFα and then protein extracts were collected from HeLa cells treated with TNFα for 0, 15, 30 or 60 minutes. Western blot analysis was carried out using antibodies against phosphorylated pERK1/2. Total ERK1/2 was subsequently probed and served as loading controls. Semi-quantitative analysis of band density was calculated for statistic analyses using one-way ANOVA. Different alphabet letters show significant differences ($P < 0.05$) of phospho-protein/total protein ratio in time course. The *Error bars* represent mean ± SE.

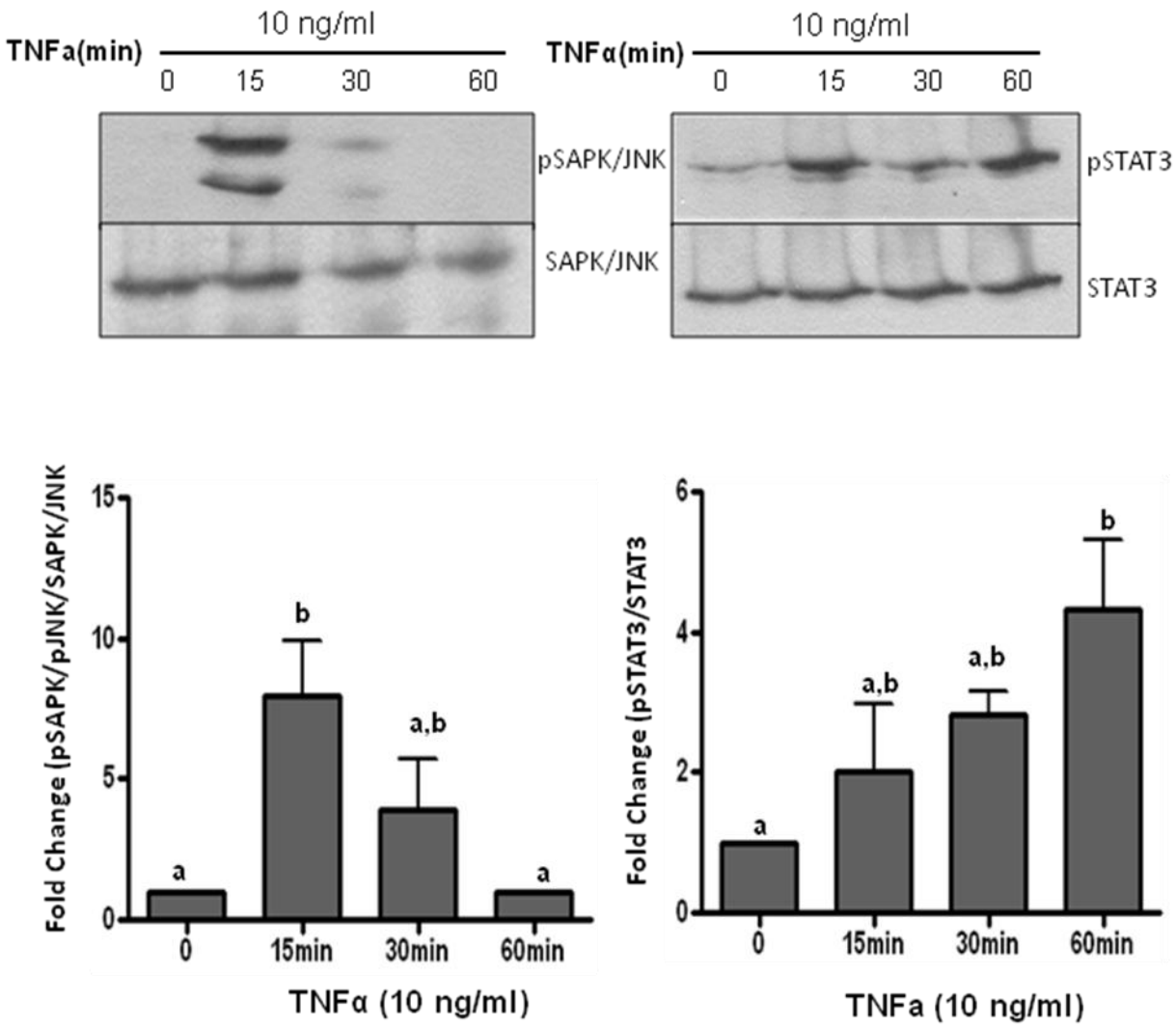

Figure 4.8. **TNFα activation of SAPK/JNK phosphorylation and STAT3 phosphorylation**. 500,000 HeLa cells were cultured in DMEM maintenance media in 10 cm dish. After 24 h serum starvation, cells were treated with 10 ng/ml TNFa and then protein extracts were collected from HeLa cells treated with TNFa for 0, 15, 30 or 60 minutes. Western blot analysis was carried out using antibodies against phosphorylated pSAPK/JNK or phosphorylated pSTAT3. Total SAPK/JNK or total STAT3 was subsequently probed and served as loading controls. Semi-quantitative analysis of band density was calculated for statistic analyses using one-way ANOVA. Different alphabet letters show significant differences ($P < 0.05$) of phospho-protein/total protein ratio in time course. The *Error bars* represent mean ± SE.

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CHAPTER 5

Summary

In this thesis, we have demonstrated that the abnormal adipocytekines play an important role in obesity associated increased cancer risk in obesity. We believe that the data in this thesis support our hypothesis that Obesity-associated adipocytokines regulate IE, and EMT genes expression profile of HeLa cells and in uterus of LY mouse.

IGF-1 activates signaling pathways of AKT, Erk1/2, STAT3 and SAPK/JNK and then regulates IE and EMT genes. Activated PI3/AKT by IGF-1 can stimulate c-Jun N-terminal kinase (JNK) and then stimulates AP-1 transcription. In addition, leptin activates signaling pathways of STAT3 and SAPK/JNK and then regulate of IE genes. However, IL-6 and TNFα can both activate signaling pathways of Erk1/2, STAT3 and SAPK/JNK and then regulate of IE and EMT genes. The activated pStat3 by IGF-1, or leptin, or IL-6 or TNFα, enhance SIS-induced enhancer of AP-1 transcription. Activation of ERK2 can cause the activation of MAPK pathway and then increase AP-1 transcription. In addition, phosphorylation of SAPK/JNK by the adipocytokines stimulates AP-1 transcription. AP-1 transcription factors enhance the induced EMT by IGF-1, or leptin, or IL-6 or TNFα. In all, the increased IE may increase the cell survival and promote tumorigensis. The activation of EMT also protects from cell death and promotes tumorigensis. Moreover, the increased pro-inflammatory cytokines including IL-6, IL-8, MCP-1 may cause inflammatory environment in obesity. The inflammatory environments may initiate or promote oncogenic transformation. All the relationships are showed in the figure 6.1.

Taken together, there is a link between adipocytokine and the expression of genes associated with cell proliferation and migration. The adipocytokine plays an important role in the initiation and metastasis of tumors in obesity. These changes in gene expression may provide a

plausible mechanism for obesity-dependent increases risk in cancers of the female reproductive tract.

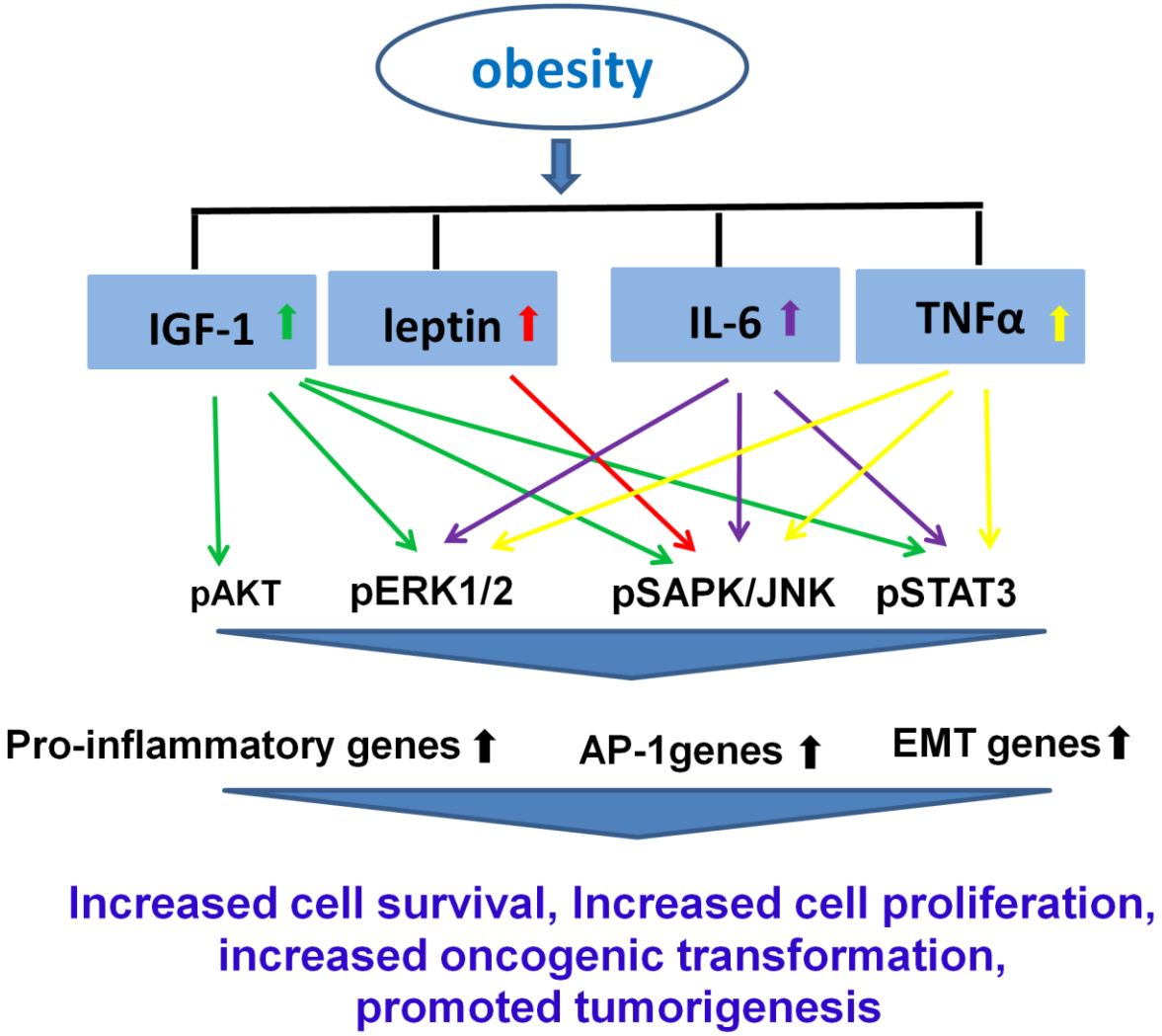

Figure 5.1. Experimental working model. The relationship between the four adipocytokins and obesity associated cancer in my experiments.